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CONTENTS

PAGE

Chemoprophylaxis against Bovine Trypanosomiasis. III. The Cure of Infected Cattle removed from a high Tsetse Density. By I. M. Smith and W. N. Scott. 325

The Pattern of Distribution of Lesions in Newcastle Disease in Northern Nigeria. By V. R. Kaschula. 343

Further Experimental Observation on Scrapie. By I. H. Pattison and G. C. Millson. 350

Q Fever and Ovine Abortion. By B. P. Marmion and W. A. Watson. 360

Haemangioendotheliomas in Poultry. By Bertil Jarplid. 370

Necrotic Enteritis in the Fowl. (Gallus gallus domesticus) I. Histopathology of the Disease and Isolation of a Strain of *Clostridium welchii*. By W. E. Parish. 377

Necrotic Enteritis in the Fowl. II. Examination of the Causal *Clostridium welchii*. By W. E. Parish 394

Necrotic Enteritis in the Fowl. III. The Experimental Disease. By W. E. Parish. 405

Adaptation of the Infectious Agent of Tick-Borne Fever to Guinea-Pigs and Mice. By A. Foggie and Catherine S. Hood. 414

The Aerobic Bacteria of the Nose and Tonsils of Healthy Dogs. By J. E. Smith. 428

Papain-Treated Red Cells in the Detection of Pig Red Cell Iso-Antibodies. By Joan R. Olds. 434

The Histopathology of the Brain of Goats Affected with Scrapie. By I. Zlotnik. 440

CHEMOPROPHYLAXIS AGAINST BOVINE TRYPANOSOMIASIS

III. The Cure of Infected Cattle Removed from a High Tsetse Density

By

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INTRODUCTION

Trypanosomes appearing in the peripheral blood of cattle under chemoprophylaxis and exposed to infection are sometimes not eliminated by a further, even an increased, dose of the drug or by related drugs (Wilson, 1953; Fiennes, 1953). Apart from its intrinsic interest, the phenomenon is important in chemoprophylactic programmes against bovine trypanosomiasis. Trials to determine the length of protection conferred on cattle by a single dose of one or other of several prophylactic drugs had been made. After they had developed parasitaemia cattle from these trials were available for observations on some aspects of this phenomenon.

MATERIALS AND METHODS

Animals. Immature, small shorthorned East African Zebu cattle came from two trials previously described (Smith, 1959; Smith and Brown, 1960). Cattle had been injected with homidium‡ suramin (B.P.) complex, R.D. 2902¶ suramin complex (Williamson and Desowitz, 1956), Prothidium§, metamidium chloride hydrochloride‖, M. & B. 4427 (fine and coarse suspensions)** and the standard preparation (British vet. Codex, 1953) of quinapyramine (prophylactic)†† Other groups, given no drugs, were also included (Tables 1 and 2). The cattle had then been exposed to infection in an area of high tsetse density, the predominant fly being *Glossina pallidipes.*

Examinations. The main observation made was the inspection of at least 200 oil-immersion fields of Giemsa-stained thick films of ear blood taken from each animal on alternate days between 07.00 and 08.30 hours (East African Standard Time). As a result all times given in days are approximate only. For diagnosis of trypanosome species in cases of doubt Leishman-stained thin films were examined.

Procedures. When trypanosomes were detected in a film from an animal (breakthrough) it was brought within two days from the infested area to a tsetse-free site.

* Present address. Trinity College, Dublin, Ireland

† Present address. Smith, Kline & French Ltd., Welwyn, Herts.

‡ Ethidium: Boots Pure Drug Co., Ltd.

¶ ———: „ „ „

§ ———: „ „ „

‖ M & B 4404: May and Baker, Ltd.

** ———: „ „ „

†† Antrycide Prosalt:Imperial Chemical (Pharmaceuticals), Ltd.

The cattle from the 1957 trial were allotted randomly to three groups for injection with either homidium* bromide or chloride (2·5 mg./kg.) or Berenil† (3·5 mg./kg.). The doses of the former two were about double the level currently used in the field (Unsworth, 1954; Milne and Robson, 1955) because at the time it was necessary to cure as many animals as possible. In the initial design of the 1958 trial the cattle were allotted by random process into groups of three as follows:—
(1.) Treatment with metamidium chloride hydrochloride (0·5 and 1·0 mg./kg.), quinapyramine‡ (4·5 and 6·5 mg./kg.), Berenil (3·5 and 7·0 mg./kg.) or homidium bromide (1·25 and 2·5 mg./kg.). For convenience in description this procedure has been called curative treatment. The lower levels of the drugs were chosen as those more approximating field levels, there being less urgency to obtain cures. (2.) A repeated treatment with the same level of prophylactic drug as originally injected. (3.) No treatment (Table 2).

No animal was injected with anti-trypanosomal drug until at least one further film containing pathogenic trypanosomes (positive film) was obtained from it. The recognised standard route of injection for each drug was used. Body weights for the purpose of calculating dosage were determined with a weighbridge accurate to about five kg. Immature, small shorthorned Zebu cattle were introduced into the herd built up of those removed from the infested area. They had been given no drug, had not previously been exposed to infection and were included in an attempt to detect the possible transmission of trypanosomes by mechanical means. The entire herd, treated, awaiting treatment, untreated and control cattle was kept in the open under range conditions, except when trypanosome counts in an individual were believed to be rising; it was then retained in a fly-proof building for a few days until the numbers decreased.

An individual was regarded as having been cured if no positive film was obtained from it during a period of examination of more than 260 days. Re-appearance of pathogenic trypanosomes in thick films, even on one occasion, in a less period was regarded as relapse, with the exception, however, that the presence of trypanosomes in films made on the first or second day after drug treatment was ignored in the compilation of relapse cases.

RESULTS

Control Animals Exposed to Tsetse.

These animals have been described previously (Smith, 1959; Smith and Brown, 1960). Of the eight from the 1957 trial, three were not treated (Table 1). They died on the average about 25 days after they were first exposed to infection. The remaining five were treated but three (67, 70, 72; Table 3), injected with either Berenil or homidium, were not cured. Nos. 70 and 72 belonged to the second batch of controls introduced into the infested area three months after the first batch, which were entered with the main herd. Possibly, therefore, resistant trypanosomes had been picked up from cattle under prophylaxis by the tsetse population, or by other biting flies

* Ethidium bromide or chloride: Boots Pure Drug Co., Ltd.
† Berenil: Farbwerke Hoechst Ag.
‡ Antrycide: Imperial Chemical (Pharmaceuticals), Ltd.

TABLE 1

TREATMENT WITH VARIOUS ANTI-TRYPANOSOMAL DRUGS

Overt infection in Zebu cattle which had been given one or other of several prophylactic drugs, exposed to infection in a tsetse area and removed from contact with the flies when overtly infected.

Prophylactic drug mg./kg. (no. of animals)	*No. + eliminated*	*Drug No. relapsed/ No. treated* HB	HC	B	*No. died negative (days)*	*Definitive No. relapsed/ No. treated*
Homidium 5 (8)	1	1/2	1/1	1/4	1 (92)	3/6
suramin complex 10 (8)	2	2/2	1/1	0/3	1 (39)	3/5
Prothidium 2 (8)o	0	0/2	2/4	0/2	—	2/8
4 (8)	1	1/2	0/3	0/2	1 (45)	1/6
RD2902 5 (8)	0	0/3	1/5	0/0	1 (60)	1/7
suramin complex 10 (8)	2	0/4	1/1	0/1	1 (77)	1/5
Quinapyramine 11·7 (16) (prophylactic)	3	0/3	0/3	3/7	2(45; 95)	3/11
Controls exposed (4)	3	0/0	0/0	1/1	—	1/1
to tsetse (4)	0	2/4	0/0	0/0	1 (64)	2/3

\+ = no. animals dead before treatment was given and animals never positive.
o = six also re-injected with Prothidium (4 mg./kg.) 38 days (average) after curative. treatment.
HB = homidium bromide (2·5 mg./kg.); HC = homidium chloride (2·5 mg./k.g.); B = Berenil (3·5 mg./kg.)

though the latter were known not to have been numerous at the time of the work, and transmitted to these animals; Fiennes (1953), Unsworth, (1954) and Lyttle (1960) observed that events of this type can occur. This does not explain relapse, however, in 67. This animal had been out of the infested area for more than two and a half months. Moreover, no cattle which had received drugs had ever been in the infested area previously. This relapse might have been related to the comparatively large number of trypanosomes present in its peripheral circulation, for Davey (1957) showed that the greater the number of *Trypanosoma rhodesiense* present in the peripheral blood of mice, the greater the amount of anti-trypanosomal drug required to cure the animals.

Before any treatment was given to the cattle removed from the infested area the contrast between the counts of trypanosomes in films from control groups and from animals which had received a prophylactic drug in which breakthrough occurred, was very noticeable. The average difference was of the order of about 750 trypanosomes/200 fields/film in the controls to about 40 trypanosomes/200 fields/film in the cattle which had been given a prophy-

TABLE 2

TREATMENT WITH VARIOUS ANTI-TRYPANOSOMAL DRUGS

Overt infection in Zebu cattle which had been given one or other of several prophylactic drugs, exposed to tsetse and removed from contact with them when overtly infected.

Prophylactic drug mg./kg. (No. of animals)	*No.° eliminated*	*Treatment* *M* 1	2	*MS* 1	2	*B* 1	2	*HB* 1	2	*P*	*Nil*	*Total+*
Metamidium 4 (30)	4	1/2	0/3	2/3	0/3	0/3	0/3	2/2	1/2	2/3	2 SC	6/21
M&B 4427 10 (30)	2	1/3	1/2	2/3	0/3	0/3	0/3	2/3	0/2	2/3	2 SC 1D	6/22
Prothidium 4 (30)	4	0/3	0/3	1/3	0/1	0/2	1/3	2/3	0/3	0/2	1SC 2D	4/21
Quinapyramine 11·7 (30) (prophylactic)	4	0/2	0/2	1/3	1/3	2/3	0/2	0/3	0/3	2/2	3D	4/21
Controls exposed to tsetse (8)	1	0/1	–	0/2	–	–	0/1	0/1	–	–	2D	0/5

o = no. of animals showing trypanosomes in one film only, died during trial, died after return from tsetse area, and died <260 days after treatment.
\+ = i.e. of animals given curative treatment. SC = self cure. D = died infected.
M = metamidium 0·5 mg./kg.(1), 1·0 mg./kg.(2). MS = quinapyramine 4·5 mg./kg.(1), 6·5 mg./kg.(2).
B = Berenil 3·5 mg./kg.(1), 7·0 mg./kg.(2). HB = homidium bromide 1·25 mg./kg.(1), 2·5 mg./kg.(2).
P = Re-injection same dose level of original prophylactic drug.
Nil = no treatment.
numerator = no. relapsed. denominator = no. treated.

lactic drug. The number of trypanosomes in thick films was widely variable and trypanosomes were not detected in every film from known infected animals after breakthrough (Smith,1959). The data revealed the existence of two major groups in these latter cattle, there being broadly in addition to those which showed consistently low average counts of ≤ 2 trypanosomes/200 fields/film, a second class with higher average counts, often as a result of a large number of trypanosomes in one or two films only (Table 4). Two of the eight controls in the 1958 trial were left untreated (Table 2) and died in a mean time of about 25 days after they were first exposed to infection. Five of the remainder were cured by treatment; the sixth died 50 days after treatment without showing any positive films.

At the times of these trials the tsetse population attacking the cattle was large (Smith and Rennison, 1961). Since these control cattle were exposed to infection at the beginning, middle and end of the 1958 trial, which lasted approximately six months, this is some evidence that, despite the apparently great likelihood of the event occurring, trypanosomes, possibly drug-resistant, were not being cyclically transmitted with any frequency, as a result of infection of the tsetse population either during the 1957 or 1958 trial. But this view, based on small numbers of animals exposed for a few days only to a vector which feeds cyclically, should not be regarded as in any way conclusive.

Prophylactically Treated Cattle Exposed to Tsetse

The total number of these cattle in the two trials was reduced for various reasons. Some were never detected as infected, others failed to show two positive films and others still died during the trials, or soon after their return to the tsetse-free area while they were awaiting treatment. The remaining cattle fell into three groups; those to which curative treatment was administered; those which received a second dose of prophylactic drug; those which were left untreated (Tables 1 and 2).

Cattle given curative treatment. Of the original 64 cattle in the 1957 trial, seven of the remaining 55 animals died without yielding a positive film from the time of treatment to the time of death, a mean period of about 65 days. They died, as far as could be ascertained, from causes other than trypanosomiasis or toxic effect of drug. Since the average period of observation after treatment was comparatively short they are not considered further. Fourteen of the remaining 48 animals relapsed after curative treatment (Table 1). Of the remaining 108 cattle of the 1958 trial, two died too soon (average about 50 days) after curative treatment to assess their response, leaving for consideration 106 animals, to 85 of which curative treatment was given with 20 relapses (Table 2). The number of relapses after curative treatment associated with each trial is not significantly different ($X^2 \simeq 0.5$, $P \simeq 0.5$).

Because of the severe challenge to which the cattle in these trials were subjected (Smith, 1959; Smith and Brown, 1960; Smith and Rennison, 1961), many, perhaps all, probably had mixed infections of all species as well as multiple infections of the same species. The thick film method of detection, coupled with a varying speed of development by each species, fails to make this clear, but the point should be borne in mind in considering all subsequent details.

Relapses arose at very variable times after curative treatment. Since there may be various interactions, e.g. drug and species, no definite period after which relapse would be uncommon can be given. Ignoring all the factors possibly involved, the mean time of relapse for both trials was approximately 60 days, with a standard deviation of about 45 days. If the distribution of relapse times was approximately normal, as appeared to be the case when they were plotted, relapse would be most unlikely after about 250 days from the time of curative treatment. The standard deviation was inflated by four or five very high values; with these omitted it was about 28 days, thus making a relapse a rare event after about 180 days. These periods are two or three times longer than that after which Whiteside (1960) regarded relapse as unlikely ($P=0.01$) in Kenya grade cattle, in which tests of drug resistance of isolated strains were made.

In the 1957 cattle *T. vivax* was found at relapse in 6, 16, 17, 30, 35, 42, 53 and 58 (Table 3). The former six had been injected with one

TABLE 3

TRYPANOSOME SPECIES DETECTED

Zebu cattle at breakthrough of several prophylactic drugs and at relapse after treatment with various anti-trypanosomal drugs.

Trial	*Animal no.*	*Prophylactic drug*	*Treatment*	*Species detected during infection*	*Species detected at relapse*	*Second treatment*	*Relapse species*	*Third treatment*
	2	HSC	B1	Tv	Tb	HC	Tc	B2 +
	6	,,	HC	,,	Tv	HB	,,	M2 +
	7	,,	HB	,,	Tc	M2	–	
	11	,,	,,	,,	,,	B2	Tc	M2 +
	13	,,	HC	Tvc	,,	HC	,,	,,
	16	,,	HB	?	Tv	B2	–	
	17	P	HC	Tv	,,	,,	,,	
1957	18	,,	,,	,,	Tc	B1	,,	
	30	,,	HB & P	Tvb	Tv	M2	,,	
	35	RDSC	HC	Tv	,,	B2	,,	
	42	,,	,,	Tc	Tvc	,,	,,	
	53	QP	B1	Tv	Tv	HC	,,	
	57	,,	,,	,,	Tb	HB	,,	
	58	,,	,,	,,	Tv	B2	died	
	67	–	,,	Tvb	Tbc	HB	,,	
	70	–	HB	Tv	Tv	HB	Tv	B2 +
	72	–	,,	,,	Tvb	HC	,,	,, o
	73	M3	M1	Tv	Tc	M2	Tc	MS2 +
	79	,,	MS1	Tb	Tb	MS2	–	
	81	,,	,,	Tv	,,	–	self cure	
	91	,,	HB1	,,	Tc	HB1	Tc	M2 +
	92	,,	,,	Tbc	Tb	M2	–	
	96	,,	HB2	Tc	Tc	MS2	–	
	98	,,	M3	Tv	,,	–	self cure	
	99	,,	,,	,,	,,	,,	,,	,,
	103	M&B 4427	M1	Tb	Tv,b(?)	,,	,, +	
	107	,,	M2	Tvbc	Tb	MS2	–	
	109	,,	MS1	,,	Tc	M2	,,	
	110	,,	,,	Tc	Tb	MS2	,,	
1958	121	,,	HB1	Tv	Tvc	HB2	,,	
	122	,,	,,	Tc	Tc	HB1	Tc	M2 +
	127	,,	M&B 4427	,,	,,	–	self cure	
	128	,,	,,	Tbc	,,	,,	,,	
	140	P	MS1	Tc	?	MS2	–	
	149	,,	B2	Tv	Tv	–	self cure	
	151	,,	HB1	Tc	Tc	M2	Tc	M3 +
	153	,,	,,	Tv	Tv	–	self cure	
	169	QP	MS1	Tvb	Tvb	M1	–	
	173	,,	MS2	Tv	Tv	MS2	–	
	176	,,	B1	Tb	Tb	B2	–	
	177	,,	,,	Tv	Tv	,,	,,	
	187	,,	QP	,,	Tvb	died infected		
	188	,,	,,	,,	,,	–	self cure	

Tv, b, c = *T. vivax, brucei* group, *congolense* + = no further relapse
? = species undetermined — = nil o = repeated relapse

1957
B1, 2 = Berenil (3·5, 7·0 mg./kg.)
HB, C = homidium bromide, chloride (2·5 mg./kg.)
HSC = ,, suramin complex
P = Prothidium
RDSC = RD2902 suramin complex
QP = quinapyramine (prophylactic)
M2 = metamidium (1·0 mg./kg.)

1958
M1, M2, M3 = metamidium chloride hydrochloride (0·5, 1·0, 4·0 mg./kg.)
MS1, MS2 = quinapyramine (4·5, 6·5, mg./kg.)
B1, 2 = Berenil (3·5, 7·0 mg./kg.)
HB1, HB2 = homidium bromide (1·25, 2·5 mg./kg.)

or other of the prophylactic drugs related to the homidium salts used for curative treatment. Thus the *T. vivax* in an animal after breakthrough of a prophylactic drug containing the phenanthridine group has apparently developed resistance to a curative drug also containing that group, but the question is why the phenomenon did not arise in all cattle so treated. *T. vivax* was also found in two cattle (53, 58; Table 3) previously injected with quinapyramine (prophylactic) and treated with Berenil after breakthrough. These cases, and the relapses after the injection of the homidium salts, occurred on the average about 50 days after curative treatment. In the 1958 trial cattle *T. vivax* was detected in 103, 121, 149, 153, 169, 173, and 177 (Table 3). This proportion does not differ significantly from the previous finding ($X^2 \simeq 1.5$, $P \simeq 0.2$). These cases arose approximately equally among cattle given one or other of the prophylactic preparations with the phenanthridine group and quinapyramine (prophylactic). Relapse arose on the average about 78 days after curative treatment. Lyttle (1960) detected resistance to Berenil in trypanosomes isolated at breakthrough of cattle under a quinapyramine (prophylactic) regime only when cattle under protection by Prothidium were also present. Since he had never been able to produce cross-resistance between Berenil and quinapyramine, Lyttle (1960) felt that these resistant strains in his field work originated from cattle under Prothidium and this assumption was strengthened by the fact that Prothidium/Berenil cross-resistance was readily produced in the laboratory. The results with 53, 58, 149 and 177 offer some confirmation of his view. Thus in field trials of prophylactic drugs conducted in the way presently employed the results with one drug may depend on the number and structure of other drugs in the trial and can, therefore, be non-independent, invalidating comparisons of protective power in part at least (Smith, 1959; Smith and Brown, 1960). Moreover, tests of other drugs against these infections either as isolated strains or in the infected animal itself, could fail to yield a true guide to the real nature of the cross-resistance developed.

Nos. 7, 11, 13, 18 and 42 (Table 3) among the 1957 cattle relapsed on the average about 75 days after curative treatment, with *T. congolense* predominating in films. All belonged to groups previously given prophylactic drugs with the phenanthridine group. In the 1958 cattle there were seven relapses (73, 91, 96, 109, 121, 122, 151; Table 3) in which *T. congolense* was found in an average time of about 15 days after treatment. Although this proportion was very similar to that observed among the cattle of the previous year ($X_c \simeq 0.1$, $P \simeq 0.8$), the negative period after curative treatment was decidely shorter. All but one case (109) arose among cattle injected with the prophylactic compounds related to homidium and treated curatively with similar drugs. Six of the seven cases were associated with the prophylactic metamidium preparations. For several reasons Smith and Brown (1960) did not exclude the chance of a drug-

resistant *T. congolense* passing among the residue of the 1958 trial cattle when they were still in the infested area. These six relapse cases were among the cattle which were found infected only later in the course of the trial. When the brief negative period after curative treatment is also taken into account, these cases could represent additional evidence of the presence of a drug-resistant *T. congolense* in the infested area during the latter part of the trial.

Two instances of relapse with *T. brucei* group were found among the 1957 cattle (2, 57; Table 3). They occurred in a mean time of about 50 days after curative treatment with Berenil. The latter is known not always to cure infection with a polymorphic trypanosome at 3.5 mg. kg. (Bauer, 1955 a, b). *T. brucei* group was found definitely in seven (79, 81, 92, 107, 110, 169, 176; Table 3) of the relapses among the 1958 cattle. The cases arose on the average 75 days after curative treatment. This increased relapse rate, which is not significant ($X_c^2 \simeq 1{\cdot}0$, $P \simeq 0{\cdot}3$), possibly reflects the presence of the prophylactic preparations of metamidium in the 1958 trial, for part of their active principle (Wragg, Washburn, Brown and Hill, 1958) is a diamidine closely related to Berenil which is regarded as rather less effective against *T. brucei* group infections. Berenil (3·5 mg./kg.) did, however, cure four cases of *T. brucei* group breakthroughs of the prophylactic metamidium preparations.

Cattle re-injected with the same prophylactic drug. Overt infection was allowed to continue until at least one definite crisis had occurred in 10 animals before they were given a second injection of the prophylactic drug at the same dose level that they had been given before exposure to tsetse. In four only were parasites eliminated (Table 2). *T. congolense* re-appeared in films from another four (98, 99, 127, 128; Table 3), re-injected with prophylactic metamidium preparations on the average about 70 days later. Films from these animals continued to be frequently positive for another 70 days or so; thereafter parasites became fewer and eventually were rarely found. Two animals (187, 188; Table 3) were re-injected with quinapyramine (prophylactic). One died eight days later with parasites always present in films in the interval. The other relapsed after 11 days. Both animals showed *T. vivax* and *T. brucei* group. Re-injection of prophylactic drug was thus comparatively ineffective after at least one crisis had taken place in the host. These findings add weight to the widely held opinion that the re-injection of a prophylactic drug must be made before peripherally circulating trypanosomes appear to avoid undesirable situations from the chemoprophylactic viewpoint.

Six animals had been re-injected with Prothidium (4 mg./kg.) for another purpose an average of 38 days after curative treatment (Table 1). A film from one contained *T. vivax* 123 days after the second dose of Prothidium. After treatment with metamidium chloride hydrochloride (1·0 mg./kg.) the animal remained negative (30, Table 3).

Untreated animals. Eleven animals were left untreated (Table 2).

Six died in an average time of about 45 days from the time they were first found infected. Clinically they were acute or subacute cases with the parasites passing through several crises until the cattle died. In others, however, average counts tended to be lower and fewer individual films contained comparatively large numbers of trypanosomes. Eventually all but one, which was not negative even at the close of the investigation, went through a period exceeding 260 days during which no trypanosomes were detected in films. Clinically these cattle were in reasonable condition. In the absence of treatment no cattle exposed to infection in the tsetse area without benefit of drugs had hitherto survived more than a few days after removal therefrom, even after exposure times of only a few hours (Smith, 1959; Smith and Brown, 1960; Smith, unpublished). Clearly prophylactic drugs exercised a modifying influence on the course and severity of the subsequent disease. The result was more particularly associated with prophylactic metamidium preparations and Prothidium, presumably because these drugs, unlike quinapyramine (prophylactic), were better able to mitigate the pathogenicity of the *T. vivax* of the infested area. With the chance of survival after removal from exposure taken as 1/100, the probability of five survivors by chance in a group of 11 is less than 1/10,000. These cattle were removed from further infection and might not have survived had they remained in the tsetse area, but it may be presumed that immune processes were taking place (Soltys, 1955) and that, as immunity developed, they might have done so.

Factors Possibly Associated with Relapse

The induced resistance in various trypanosomes to drugs ranges from slight to high (Schnitzer and Grunberg, 1957). Strains isolated from cattle in the course of chemoprophylactic trials show this varied response (Williamson, 1960). Although the numbers of cattle described in this paper are small and the indications inconclusive as a consequence, they also offer some suggestion of the relationship of response to prophylactic and curative drug and trypanosome.

Under the conditions of the work and ignoring the drug used in later treatment, each prophylactic drug appeared to give a more or less similar proportion of relapses ($X^2 \simeq 6{\cdot}4$, $P \simeq 0{\cdot}5$, Tables 1 &2; see also Table 6). The results for the 1957 and 1958 trials with Prothidium and quinapyramine (prophylactic) are useful in that they suggest that there could have been no more than a minimum build-up of drug-resistant strains in the tsetse in the infested area during and between the trials, which had an interval of about three months between them. This conclusion is without doubt also influenced by the factors which govern the size and structure of tsetse populations. The relative proportions of relapse species (Table 5) was also similar. Relapse was thus apparently a phenomenon not closely connected with either one particular prophylactic drug or one species of trypanosome.

Relapse was, however, apparently associated with the drug used in curative treatment (Tables 1 & 2). Relapse occurred with all, but proportionally more occurred with quinapyramine (4·5 mg./kg.) and homidium bromide (1·25 mg./kg.); these levels would probably be unreliable in the field in arresting overt infection in cattle under protection by the range of prophylactic drugs given here. Of the six relapses in which quinapyramine (4·5 mg./kg.) was employed curatively, four (79, 81, 109, 110; Table 3) arose in cattle given originally metamidium preparations prophylactically. While quinapyramine (4·5 mg./kg.) thus appears unsatisfactory for use against breakthroughs of prophylactic metamidium salts, this observation is inconclusive because in three of the four cases *T. brucei* group was involved. Quinapyramine (4·5 mg./kg.) may be rather ineffective against this clinically comparatively mild pathogen (Davey, 1957). Relapses after homidium treatment (1·25 mg./kg.) arose in six cattle (91, 92, 121, 122, 151, 153; Table 3) originally given prophylactic drugs with the phenanthridine group as part at least of the active principle. This type of event is well known in both field and laboratory (Whiteside, 1960). All the recognised trypanosome species of the infested area were represented in these relapses. In the cattle from the first trial even the higher level (2·5 mg./kg.) of homidium salts was comparatively ineffective with 10 relapses out of 30 cattle curatively treated (Table 1), but in second trial there was only one relapse out of ten animals treated with homidium (2·5 mg./kg.) as shown in Table 2. Although not significantly variable ($X_c^2 \simeq 1{\cdot}0$, $P \simeq 0{\cdot}3$) the two responses are suggestive and probably reflect the preponderance of closely related phenanthridiniums as prophylactic drugs in the first trial compared with the preponderance of less closely related compounds in the second. There was also some indication (Tables 1 & 2) that metamidium (0·5 mg./kg.) and Berenil (3·5 mg./kg.) might prove inadequate in the control of breakthroughs of this range of prophylactic drugs. Although quinapyramine (6·5 mg./kg.), Berenil (7·0 mg./kg.) and metamidium (1·0 mg./kg.) were comparatively reliable in eliminating pathogenic trypanosomes at breakthrough of prophylactic drugs with the phenanthridine group, no curative drug, as used by the methods given here, was completely effective (Tables 1 & 2). Any drug except itself and Berenil (3·5 mg./kg.) was useful for breakthrough of quinapyramine (prophylactic). The comparatively great effectiveness of Berenil (3·5 & 7·0 mg./kg.) (Table 2) in treating breakthroughs of the prophylactic metamidium preparations is unexpected because of the relationship between them. A similar result has been reported by Stephen (1960).

The preceding observations regard the situation as one involving only the interaction of the pathogen with the prophylactic drugs and the agents used for curative treatment. As shown, however, a number of cattle recovered even without treatment. Trypanosome counts in animals before treatment indicated broadly the existence

of two classes of cattle and this feature was more apparent in the counts of the cattle which were later curatively treated. Nearly half of the animals had average counts of about two or fewer trypanosomes/200 fields/film (Table 4). The 133 cattle curatively treated were classified as in Table 6. The appended analysis shows that a successful outcome to curative treatment was dependent ($P < 0.01$) upon a low average count and only on this

TABLE 4

DISTRIBUTION OF COUNTS OF TRYPANOSOMES

Thick blood films from Zebu cattle exposed to a high density of tsetse

Year of original trial of prophylactic drug	*Grouping*	*No. animals with given average counts* ⩽ 2	3–4	5–10	11–50	> 50
1957	controls exposed to tsetse	0	0	1	0	7
	prophylactically injected cattle	23	4	7	7	7
1958	controls exposed to tsetse	0	0	0	0	8
	prophylactically injected cattle :					
	1. curatively treated	40	12	12	15	6
	2. prophylactic repeated	1	0	1	6	2
	3. untreated *	2	1	3	1	3

* one animal not classified because positive beyond 260 days.

TABLE 5

DISTRIBUTION OF SPECIES OF PATHOGENIC TRYPANOSOMES

Zebu cattle injected with prophylactic drugs, exposed to tsetse and treated with various drugs after removal from contact with tsetse when overtly infected

Prophylactic drug		*Trypanosome species** T. vivax *No. cases detected*	T. vivax *No. relapses*	T. brucei *No. cases detected*	T. brucei *No. relapses*	T. congolense *No. cases detected*	T. congolense *No. relapses*
Homidium suramin complex		8	2	1	1	2	3
Prothidium	Trial 1957	13	2	1	0	1	1
	1958	16	2	2	0	6	1
RD 2902 suramin complex		10	2	0	0	1	1
Metamidium chloride hydrochloride		13	0	9	3	8	5
M&B 4427		11	2	9	3	12	5
Quinapyramine (prophylactic)	Trial 1957	10	2	0	1	1	0
	1958	20	5	4	4	0	0

*mixed infections shown against each species and undetermined infections (8) ignored.

factor among those considered. Counts were not associated particularly either with the duration of protection from prophylactic drug or with the length of time an animal was positive before curative treatment was given. It is also clear that neither the length

TABLE 6

DISTRIBUTION OF 133 INFECTED ZEBU CATTLE

Treated in a tsetse-free area with several anti-trypanosomal drugs after breakthrough of various prophylactic drugs in a tsetse area

Prophylactic drug	*Mean duration prophylaxis*	*Mean time before treatment*	*Approx. mean trypanosome count*	*Result of curative treatment:* *Cure*	*Relapse*
Homidium suramin complex	not exceeded	not exceeded	<3	1	0
			⩾„	1	3
		exceeded	<„	0	0
			⩾„	1	1
	exceeded	not exceeded	<„	0	0
			⩾„	1	1
		exceeded	<„	0	0
			⩾„	1	1
Prothidium	not exceeded	not exceeded	<3	7	0
			⩾„	4	3
		exceeded	<„	5	1
			⩾„	0	1
	exceeded	not exceeded	<„	5	1
			⩾„	5	0
		exceeded	<„	2	1
			⩾„	0	0
RD 2902 suramin complex	not exceeded	not exceeded	<3	2	0
			⩾„	1	1
		exceeded	<„	2	0
			⩾„	0	0
	exceeded	not exceeded	<„	0	0
			⩾„	1	1
		exceeded	<„	4	0
			⩾„	0	0
Quinapyramine (prophylactic)	not exceeded	not exceeded	<3	2	2
			⩾„	2	1
		exceeded	<„	6	1
			⩾„	3	3
	exceeded	not exceeded	<„	5	0
			⩾„	3	0
		exceeded	<„	1	0
			⩾„	3	0

Table 6 continued

Prophylactic drug	*Mean duration prophylaxis*	*Mean time before treatment*	*Approx. mean trypanosome count*	*Result of curative treatment: Cure*	*Relapse*
Metamidium chloride hydrochloride	not exceeded	not exceeded	<3	3	0
			⩾„	3	1
		exceeded	<„	2	1
			⩾„	1	0
	exceeded	not exceeded	<„	0	0
			⩾„	3	3
		exceeded	<„	1	1
			⩾„	2	0
M & B 4427	not exceeded	not exceeded	<3	2	0
			⩾„	4	1
		exceeded	<„	2	0
			⩾„	1	0
	exceeded	not exceeded	<„	4	0
			⩾„	1	2
		exceeded	<„	1	0
			<„	1	3

ANALYSIS

(transformed data)*

Source of variation	d.f.	mean sq.	F
Main plot (Drugs (D), duration prophylaxis (P) times before treatment (L), counts (C) and error)	47	12·3871	—
Cure or relapse (X)	1	5·3582	40·24*xxx*
DX	5	0·2921	2·19
PX	1	0·0140	<1·00
LX	1	0·1854	1·39
CX	1	1·2650	9·52*xx*
DPX, DLX, DCX, PLX, PCX, LCX⁰	18	0.9169	—
Error	21	0·1328	—

* no. of animals (n) cured/relapsed in each subgroup transformed as $x = \sqrt{n + 0{\cdot}5}$; $x_1, x_2 \ldots$ used for analysis

⁰ individually not significant $xxx = P < 0{\cdot}001$ $xx = P < 0{\cdot}01$

of time an animal had been overtly positive before curative treatment nor the length of prophylaxis affected the result of this treatment. Consequently the situation seems more reasonably representable as an interaction of four factors, viz. host, prophylactic and curative

drug, and trypanosome. It might be held that these relapse cases were not cured for the same reasons as the relapses among control cattle (Table 1). The average number of trypanosomes/200 fields/film was about 750 in the control cattle exposed to tsetse and about 45/200 fields/film in the prophylactically injected cattle which relapsed after curative treatment. This sixteen-fold difference may imply a different reason for relapse.

Subsequent Relapses

These results can be seen in Table 3. Of the 14 relapses from the first trial four (2, 6, 11, 13) relapsed again despite a second curative treatment. In each case recrudescence of *T. congolense* occurred on the average about 72 days after this treatment. Either Berenil or metamidium (7·0 mg./kg. and 1·0 mg./kg. respectively) was used in an attempt to cure these individuals. No third relapse was detected. In the animals from the 1958 trial four also relapsed a second time with *T. congolense* in an average period of about 13 days, despite repeated curative treatment. For each case homidium or metamidium had been used. All had been injected originally with prophylactic drugs containing the phenanthridine group. After further treatment with metamidium or quinapyramine no subsequent relapse was detected (73, 91, 122, & 151).

Cattle without Drugs not Exposed to Tsetse

No film containing pathogenic trypanosomes was ever obtained from these eight animals.

Self Cures

Nine cattle (81, 98, 99, 103, 127, 128, 149, 153, 188; Table 3), which relapsed after curative treatment or after re-treatment with prophylactic drug, showed trypanosomal counts gradually decreasing in number and fewer positive films until no further trypanosomes were detected during a period of examination exceeding 260 days. Four were cases of *T. congolense* infections, one *T. brucei* group, two *T. vivax* and two mixed. These cases probably represent the same type of response which has already been considered in detailing the untreated cattle which had originally been given prophylactic drug.

DISCUSSION

In African bovine trypanosomiasis apparent relapses may be, in fact, re-infections. In our cases re-infection can probably be discounted on the following grounds. (1) Control cattle, which had not received any drug, introduced in the herd in the tsetse-free area were never detected as infected. (2) The comparatively low numbers and fleeting appearances of trypanosomes in the peripheral blood of infected cattle probably militated against mechanical transmission by a population of blood-sucking flies which was known

to be low in the tsetse-free area; the possibility of such transmission was further reduced by periodical spraying intended primarily to reduce tick infestation. (3) Each pin used for bleeding was discarded after use on one animal. (4) It was difficult to obtain a transmission by removal of blood from the ear veins and subsequent injection into other animals, including sheep.

It seems safe, therefore, to regard the re-appearance of parasites in some of the cattle as evidence of relapse and not re-infection. The subsequent findings must not be regarded as necessarily indicative of the likely course of events in the field because the cattle were removed from further risk of infection almost as soon as overt infection was observed. In addition, the number of variables which may exist seriously compromises any conclusions to be derived from the small numbers of cattle available. The data also exhibit the other serious drawback in collating and interpreting observations on trypanosomal chemotherapy, namely, the element of unpredictability (Schnitzer and Grunberg, 1957).

Despite many investigations the basic mechanism by which trypanosomes acquire drug resistance is obscure (Schnitzer and Grunberg, 1957; Bishop, 1959; Williamson, 1960). In the absence of this vital information, a discussion of the reasons for relapse can be considered under three headings.

1. The spectrum may be inadequate. It was well established by Davey (1957) that a given curative drug may be comparatively ineffective against an individual species of trypanosome e.g. quinapyramine and *T. vivax*. The same phenomenon is noted in the results in this paper but, in general, this view would account for only part of the findings, for some 70 per cent of the cattle involved were cured.

2. A real resistance may be produced. Some cattle under prophylaxis which are overtly infected are clearly harbouring trypanosomes which can resist doses of drugs which otherwise would cure. A true resistance may have arisen not necessarily in the individual per se but rather following infection in the infested area by drug-resistant trypanosomes from other cattle. Trypanosomes can be detected in the blood of cattle which have had no drug by the inoculation of blood into small animals some days before the individual is detected as infected by the thick film method of diagnosis (Smith, Scott and Rennison, unpublished), and so tsetse could be infected before the animal was removed from contact with them. It has not been demonstrated in the field that there is an initial period during which trypanosomes entering a prophylactically treated host cannot survive in it, prophylactic drugs being no more than suppressives (Fiennes, 1953; Robson and Milne, 1957). Thus the possibility of infection of tsetse with possibly selectively drug-resistant trypanosomes might be greatly increased. Relapses could be a result of such infection. In our work, however, had there been drug-resistant trypanosomes in the tsetse population, a shorter

duration of protection by some of the prophylactic drugs and a higher proportion of relapses in the second trial would have been expected.

3. It is possible that the host may play a part. If applicable to other species in other hosts, the finding of Davey (1957) on the relationship of the numbers circulating and the amount of drug needed to cure may explain relapse in some cases. This feature may not be quantitative (Whiteside, 1957) but qualitative. With a single strain of *T. equiperdum* the number of immunologically detectable mutants is about two per million trypanosomes per generation of the latter (Cantrell, 1958). If the species pathogenic for cattle mutate at a similar rate multiple infections of the same species, such as probably occurred in the cattle in these trials, would produce a serious rise in the number of variants in the individual against which protection has to be established. Thus the host itself could play an important part in the outcome of treatment. This role was once given prominence (Mesnil and Brimont, 1908; Jancsó and Jancsó, 1934) but has also been discounted (Yorke and Hawking, 1932). As Soltys (1959) pointed out, much can depend on the species of host. Although *T. congolense* relapses were most common, Calver (1945) working with *T. congolense* in mice found true resistance to the phenanthridinium compounds most difficult to establish. She found the organism resisted members of the series progressively through four categories:– the strain at acme, early infections, a "chronic" strain at acme in another host, and the "chronic" strain in the same host. Soltys (1959) clarified these findings when he indicated that once antibody resistance was established in a relapse ("chronic") strain (*T. brucei*), resistance to drug concommittantly increased. In our cattle the likelihood of cure tended to be associated with low trypanosomal counts, i.e. crises had probably not occurred, the animals resembling those in the second category of Calver (1945). Once a crisis had taken place the likelihood of cure was seriously reduced, i.e. such animals would fall into the fourth category and were harbouring trypanosomes which were the most difficult to deal with, not because they were resistant in the usual sense to the drugs employed, but rather because, being antibody resistant, they were refractory to the hosts' defence mechanism. As a corollary to this conjecture conclusive tests in new hosts of the resistance to drugs of strains isolated from cattle under prophylaxis must depend largely on a history of the isolates sufficiently detailed to determine the possible presence of antibody resistance where this cannot be strictly measured. Such information is not always readily available. The issue raised by the work of Calver (1945) and Soltys (1959) is whether or not their findings, made with old non-cyclically passaged laboratory strains, also occur with cyclically transmitted organisms in the field; our observations suggest that they may. However, since repeated contact of an antibody-resistant strain with drugs presumably renders it more and more drug-resistant, in the practical sense it is irrelevant whether

the trypanosomes are resistant to drug or antibody or both. The sanative concept (Whiteside, 1958) seems valid, therefore, in the field. On the other hand undetected crises may have occurred in the animals with low average counts and they were, therefore, approaching premunity so that many might have survived without treatment. Although re-injection of prophylactic drug did not improve the survival rate, the initial single dose of prophylactic drug apparently had moderated the infection which occurred later to an extent which permitted some animals to survive. Many cures obtained after therapy might thus have been obtained without it, though probably less directly. Consequently, there seems as yet insufficient evidence for categorical statements that a given drug cures breakthroughs of a given prophylactic drug in the field.

CONCLUSIONS

Immature, small, shorthorned East African Zebu cattle, which had been given a single dose of one or other of various chemoprophylactic drugs against trypanosomiasis, were removed from contact with tsetse (chiefly *G. pallidipes*) when pathogenic trypanosomes of the *T. vivax*, *T. congolense*, *T. brucei* group species were detected in their peripheral blood: they were divided into three groups.

Among those allotted to a group for treatment with several antitrypanosomal drugs (homidium, 1·25 and 2·5 mg./kg.; Berenil, 3·5 and 7·0 mg./kg.; quinapyramine, 4·5 and 6·5 mg./kg.; metamidium, 0·5 and 1·0 mg./kg.) relapse after treatment occurred in about 30 per cent. The most efficacious drugs were Berenil (7·0 mg./kg.), metamidium (1·0 mg./kg.) and quinapyramine (6·5 mg./kg.). No drug of those employed was completely effective. As well as being associated with the drug used therapeutically, the likelihood of cure was apparently also dependent upon the occurrence of comparatively low average trypanosomal counts in thick blood films before chemotherapy.

Re-treatment of a second group, in each member of which at least one crisis had taken place, with the same prophylactic drug which they had received originally directly eliminated infection in 40 per cent only, though another 40 per cent also survived with eventual self cure.

A considerable proportion (45 per cent) of a third group which were given no therapy survived, indicating a considerable modification by chemoprophylactic drugs of the virulence for cattle of the trypanosomes of the infested area, which killed untreated controls in less than one month.

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THE PATTERN OF DISTRIBUTION OF LESIONS IN NEWCASTLE DISEASE IN NORTHERN NIGERIA

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INTRODUCTION

Newcastle disease has been by far the most important epizootic poultry disease in Northern Nigeria during the past three years. It usually appears during the dry season of November to April each year. It takes a highly virulent form and causes heavy mortality in village flocks. As there are two main types of Newcastle disease in other parts of the world—the mild American form and the virulent Asiatic type—it is necessary to differentiate between these two before different virulent forms of the disease are discussed.

The mild form of Newcastle disease is prevalent in America, causing a low mortality rate in adult birds but a higher death rate in young chicks. It can, however, cause considerable losses in production and also such sequelae as respiratory disorders or nervous complications. The principal lesion at necropsy is an aerocystitis, which may, however, be secondary to the viral infection. Alimentary tract lesions are seldom seen. This disease is characteristically air-borne and appears to be spread mainly via the respiratory system.

In most tropical and sub-tropical countries per-acute or acute virulent forms are seen, which cause a high mortality. The principal macroscopic lesions are in the alimentary tract, where haemorrhagic or ulcerative lesions are to be found, but characteristic histological lesions occur in the central nervous system (Sullivan, 1958), which consist of an extensive hyperaemia, accompanied by proliferation of the endothelial cells. These nervous lesions correspond with the common clinical picture of CNS involvement, such as:- bilateral twitching of the head and wings; marked weakness of the limbs; respiratory difficulty, which appears to be a nervous failure rather than due to organic lesions in the respiratory system; inco-ordination, tremor and the resulting torticollis and paralysis of the limbs or viscera. This virulent form appears to be spread by the alimentary tract, probably through the drinking water.

In pigeons the virulent experimental form produces nervous symptoms as the main feature of the infection (Doyle, 1927). The virus of the mild American form produces a reaction only if injected intracerebrally, whereas the virus of the virulent form will produce the disease if given by intramuscular or subcutaneous injection. The above comparison applies to the mild and virulent forms in general.

Since this article deals with the virulent variety present in Nigeria, it is appropriate to discuss the different types of the virulent disease found elsewhere. Doyle (1927), in his first cases in England, described an obviously exotic disease which, although rather like fowl plague, differed in that lesions were far less conspicuous. Only the proventricular lesion in the alimentary tract was regarded as being characteristic, but a general enteritis was present. The disease described in the Far East,

resembled fowl plague even more closely (Kraneveld, 1926; Crawford, 1930; Albiston and Gorrie, 1942). The disease encountered in South Africa by Kaschula, Canham, Diesel and Coles (1946) was characterised by a diphtheritic pharyngitis in addition to the usual intestinal and proventricular lesions.

The form of the disease prevalent in Nigeria is described in this article. It seems to resemble the Asiatic variety most closely. The intestinal lesions are characterised by haemorrhagic necrotic lesions in the proventriculus, the intestinal mucosa and the caeca, and are confined to well defined areas.

MATERIALS AND METHODS

More than 500 autopsies were performed, largely on birds dying in natural outbreaks in imported White Leghorn and Rhode Island Red, as well as in native birds. All ages and sexes were represented. About 100 birds infected orally with an egg-propagated virus were also examined.

The method of studying the distribution of lesions along the alimentary tract was as follows:- Maps of the size and distribution of the intestinal lesions were drawn on a length of plank 4 feet long and one inch wide. The largest chicken dealt with had intestines 4 feet long, and the average length was between 2 and 4 feet. Diagrams were therefore prepared for intestines 2, 3 and 4 feet long. When an intestine was being studied it was stretched out on the plank and the distribution of the lesions was compared with the diagram nearest its length. In this way a rapid approximate comparison was made.

RESULTS

Observations based on cases resulting from both natural and artificial exposures have indicated that if deaths occur 3 to 5 days later, classical lesions and symptoms are usually evident. In cases with a longer illness, less definite lesions and symptoms are seen, but in a fairly high percentage of survivors torticollis and other characteristic nervous symptoms are seen. The necropsy findings are classical in the acute form, but vague in the subacute and negative in chronic cases.

The necropsy findings of the disease in Nigeria were as follows. Enlargement of the spleen was particularly evident in the most acute cases and the organ was twice the normal size. This lesion has been seen by others (Todd, 1954; Beaudette, 1956). A small or shrunken spleen was characteristic of subacute and chronic cases. Congestion and acute inflammation of the kidneys was usually present in acute cases. Petechial haemorrhages sometimes occurred in the musculature, heart, pleura and mesentery and somewhat resemble the lesions in fowl plague, but were not constant or outstanding lesions. In acute cases, haemorrhagic and diphtheritic lesions which sometimes become ulcerative were consistently present in the alimentary tract.

In addition to the proventricular and caecal lesions, ten other main sites of lesions in the mucosa and submucosa of the intestinal

tract were seen at definite locations. They are illustrated in Fig. 2 and are distributed as follows:-

Diffuse areas of intense reddening of the duodenum occur where it joins the gizzard (Figs. 2 & 3 lesion 2), and also in the cloaca (Figs. 2 & 3, lesion 12).

Oblong, well defined and discrete lesions occur along the course of the intestinal tract in a definite pattern, labelled 3-10 in Figs. 2 & 3. In Fig. 1 an attempt has been made to demonstrate the

Fig. 1.

The opened alimentary tract of two birds shown side by side to illustrate the similar distribution of the lesions at 10 of the 12 sites shown in Fig. 3.

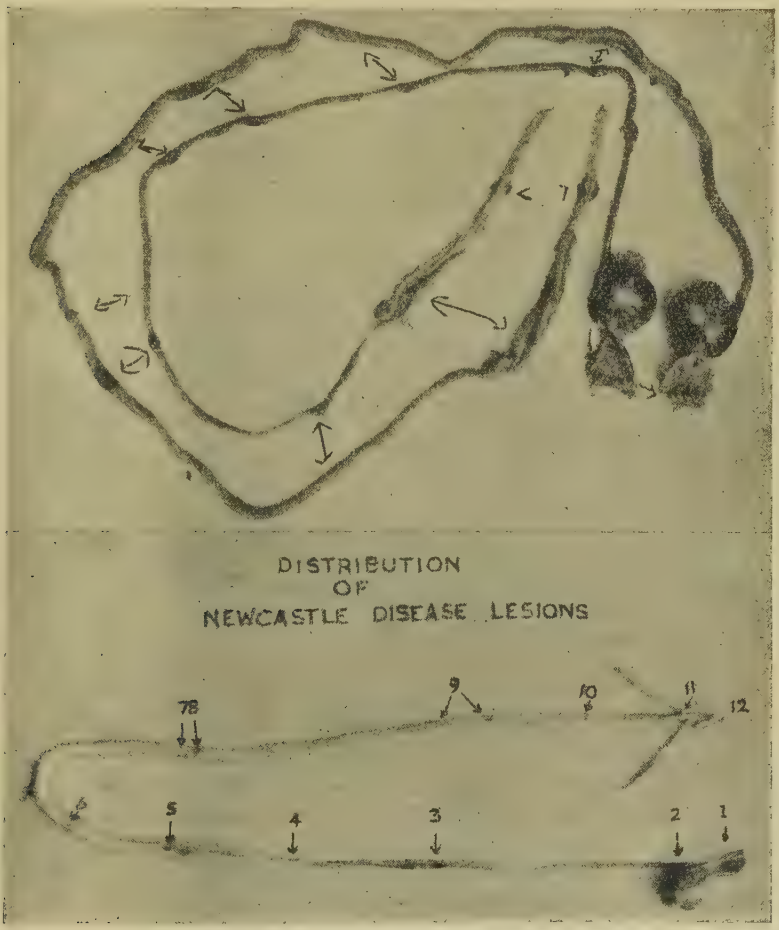

Fig. 2.

The distribution of the lesions in the opened alimentary tract in acute Newcastle disease.

similarity in distribution of the lesions in one intestine with those of another. In Fig. 3 the relative location, size and distribution of the main lesions are illustrated diagrammatically. These lesions are bluish-red in colour and are raised above the surface of the mucosa, but they penetrate into the submucosa of the intestinal wall and are visible from the visceral surface. The lesions are diphtheritic in nature. They vary in size and shape from one another, but the location, size and shape of the lesions in any one site is approximately constant. Sometimes the lesions are not single but broken into a number of smaller ones (Figs. 2 & 3, lesion 9).

Fig. 3.

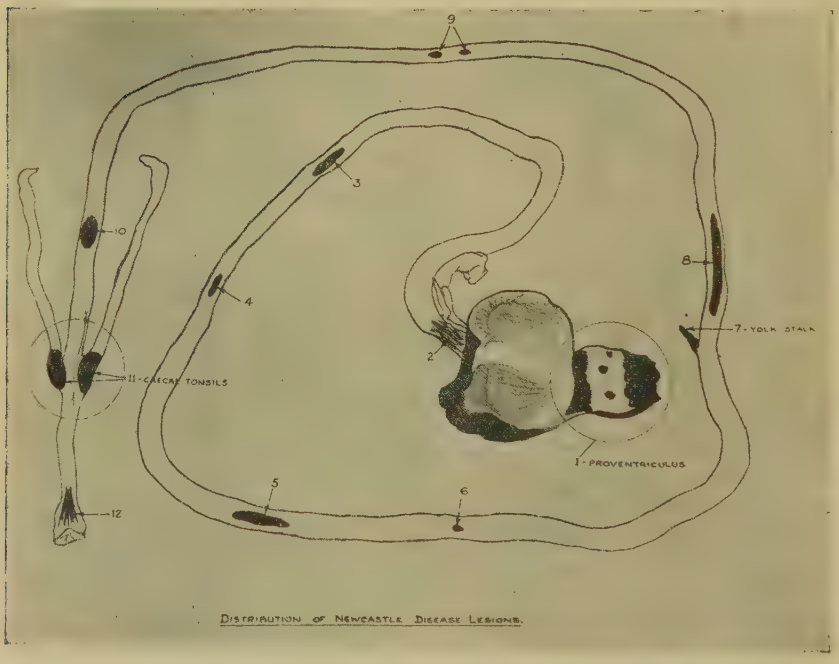

Diagram of the sites of the 12 lesions seen regularly in the alimentary tract.

These lesions are seen only in peracute and acute cases and are absent in chronic cases. This is unfortunate from a diagnostic point of view, since only early cases are usually characteristic and the deaths occurring a few days after the commencement of mortality often give negative findings. If early cases can be secured, a quick diagnosis can be made from the appearance of the lesions, and immediate control measures can be applied

In about one per cent of cases, in addition to the lesions described above, numerous pinpoint haemorrhages may be seen in the mucosa and submucosa of the intestinal wall and in the fat, particularly around the heart, proventriculus, gizzard and mesentery, and in

the musculature, especially along the course of the sciatic nerves. These cases resemble classical fowl plague and may also be mistaken for peracute fowl cholera.

Pinpoint haemorrhages, hyperaemia or catarrhal inflammation can be seen in the tracheal mucosa, but these lesions are not highly characteristic. In the peracute form intestinal lesions may be absent or ill-defined but marked reddening of the yolk stalk is present and swelling of the spleen is seen, (Figs. 2 & 3, lesion 7).

The lesions described above are generally clear-cut in birds dying 5 days after exposure. The longer the birds take to die the less likely are the lesions to be seen, so that while a fair percentage of those dying at 6 days have classical lesions, those dying on the 7th to 9th days are likely to be negative. The lesions in the proventriculus are more consistent but are seldom present as long as 9 days after exposure. The nature of the lesions changes from a haemorrhagic, diphtheritic or necrotic lesion in acute cases to an ulcerative lesion in less acute ones.

Small and pale spleens are characteristic of the subacute and chronic disease. The kidneys show lesions in subacute and chronic cases, but not in peracute ones. In chronic cases there is macroscopic evidence of nephritis, with congestion of the ureters and tubules with urates. Very occasionally there are extensive deposits of urates on the viscera (visceral gout).

In birds dying 10 days or more after exposure it is usual to find a negative postmortem picture, but only a small proportion survive to this stage. The few survivors develop various forms of nervous disorders such as torticollis, inco-ordination, paralysis of the limbs, or dysfunction of certain viscera due to nervous involvement.

The virulent form described here differs from the mild American form which is characterised by respiratory and nervous symptoms. In the latter the only macrosopic lesions are found in the respiratory system in which thickening of the airsacs (aerocystitis) is the most noticeable lesion, and this lesion is apparently due to secondary invaders. There is usually a complete absence of alimentary lesions in the American form.

DISCUSSION

It has been recognised by many workers that the haemorrhagic necrotic lesions in the proventriculus and in the caeca appear in well defined areas (Doyle, 1927; Crawford, 1930; Orr and John, 1946; Jungherr *et al*, 1946; Hecke, 1944). Jungherr *et al* (1946) stated that haemorrhages and secondary necrosis occur in association with the single plaques in the lower intestinal canal, but did not indicate that these lesions occur in a well defined pattern or that the pattern of one case resembles that of another case of similar acuteness. The findings in the cases studied in Nigeria, however, indicate that this is so. The illustrations of intestinal lesions in the

literature (Jungherr *et al*, 1946; Todd, 1954) and the author's observation of the virulent disease in South Africa and America, suggest that the pattern is probably the same in these countries as well.

The appearance of intestinal lesions at constant sites raises the question of whether lymphoid tissues are involved. Jungherr *et al* (1946) regard the haemorrhagic and necrotic involvement in the intestines as being present near to or in the lymphoid plaques and de Kock (1954) also considers the lesions to be present in the lymphoid tissue. According to Dransfield (1946) there are no lymph glands in the chicken in the sense that they occur in mammals. Lymphoid plaques and plexuses occur in the intestinal mucosa, but their distribution has not been carefully mapped. It seems likely that the scatter of the lesions in Newcastle disease corresponds with the distribution of the lymphoid plaques.

The appearance of hyperaemia, haemorrhages and so-called endotheliosis, with an infiltration of the capillary walls with lymphocytes and other blood cells, has been noted in the CNS by various authors, (Jungherr *et al*, 1946; Karzon and Bang, 1951; de Kock, 1954). Sullivan (1958) states that in the acute cases of the disease the predominant tissue change is an extensive hyperaemia of the CNS, accompanied by proliferation of the endothelial cells. This probably explains the nervous symptoms in the disease.

In about one per cent of cases haemorrhages occur in the fat and musculature, and these lesions resemble those of fowl plague (Todd, 1954). These and other lesions in the CNS suggest that the degeneration is primarily in the blood capillaries, but that lymphoid tissue is also prone to damage.

Comparison of the lesions in Newcastle disease with those in similar diseases of other animals is interesting. Discrete areas of necrosis and inflammation occur in the mucosa of the intestine in hog cholera and African swine fever and in the lymphatic glands that are located in the area (Henning, 1956). The lesions in these diseases, however, differ in certain important respects. In Newcastle disease they are only seen in peracute cases, whereas in the two pig diseases they are more pronounced in the Peyer's patches in subacute cases. The haemorrhagic lesions in the lymph glands are very prominent in the peracute forms, and less so in subacute cases. It would seem that the "button ulcers" in the Peyer's patches are due to secondary infection of necrotic lesions, created in the first place by the virus itself.

The existence of an enlarged spleen in virus infections is not often encountered, but it is seen in peracute forms of certain diseases. It is usually present in the most peracute forms of African swine fever (Henning, 1956) and hog cholera but not in the less acute forms, particularly of hog cholera. It is also seen in peracute equine infectious anaemia (Stein and Mott, 1947) but is absent in the less acute form.

CONCLUSIONS

The different forms of Newcastle disease and the principal macroscopic lesions in the acute, subacute and chronic forms are described.

In a study of the acute form of Newcastle disease in Northern Nigeria it has been found that in addition to the necrotic patches in the proventriculus and the caeca, there are lesions in the wall of the intestines. The pattern of distribution of these lesions in any one site is very similar in birds dying at the same stage of the acute disease.

Peracute and acute cases usually show classical lesions, including swelling of the spleen, but this and the intestinal lesions become less conspicuous in subacute cases and they are absent in chronic ones.

The literature dealing with the appearance and structure of the lesions is discussed.

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FURTHER EXPERIMENTAL OBSERVATIONS ON SCRAPIE

By

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INTRODUCTION

The literature on scrapie up to 1958 has been reviewed by Palmer (1959). More recent papers are those of Chandler (1959), Hadlow (1959a, 1959b), Moulton and Palmer (1959), Pattison, Gordon and Millson (1959), Stamp, Brotherston, Zlotnik, Mackay and Smith (1959), Avery, Mills and Darcel (1960), Darcel and Avery (1960), Gordon (1960), Gordon, Stamp, Shahan, Hadlow and Hourrigan (1960), Mackay, Smith and Stamp (1960), Millson, West and Dew (1960), Pattison and Millson (1960), Parry (1960), Stamp (1960), Wight (1960), Zlotnik (1960), Wight (1961), Pattison and Millson (1961a, 1961b). Many workers have attempted to characterise the causal agent of scrapie, but no agreement has yet been reached regarding its nature or mode of action.

In this paper a description is given of further attempts to characterise the scrapie agent and certain similarities between this agent and that associated with experimental allergic encephalomyelitis (Kies and Alvord, 1959) are discussed.

MATERIALS AND METHODS

Experimental Animals

Goats were used for all experiments. Some of these were bred at Compton and some were bought as kids a few days old. All were reared in a non-scrapie environment until such time as they were required for experment. Details regarding sex, age, and whether Compton-bred (C) or bought-in (B) are given under the descriptions of each experiment.

Preparation of Materials for Inoculation

All inoculations were of 1·0 ml. given intracerebrally into the substance of the brain. The various materials inoculated were as follows:—

Normal brain suspension. A ten per cent w/v suspension in physiological saline was prepared by grinding in a mortar with a little sand a weighed amount of brain from a freshly killed goat, adding the requisite amount of saline, and centrifuging at 1,500 r.p.m. for 15 minutes. The supernatant constituted the inoculum. Inocula were held overnight at 4°C before use.

Cerebrospinal fluid. C.S.F. was obtained by cisternal puncture under Nembutal anaesthesia.

Heated brain suspension or C.S.F. Heating of the supernatant of a ten per cent w/v suspension of scrapie brain in physiological saline or phosphate buffer saline, or of undiluted C.S.F. was carried out by placing 3·0 ml. amounts in 5·0 ml. glass ampoules, or 6·0 ml. amounts in 10 ml. glass ampoules, sealing in vacuo and immersing these in boiling water for the requisite period. Material described as autoclaved was subjected to approximately 15 lb. steam pressure for 15 minutes. A small laboratory autoclave was used, and the time that elapsed between placing the ampoules in this apparatus and recovering them again after heating was about one hour. The scrapie brain suspension used for this work was prepared from two

scrapie goat brains held for 24 hours at 4°C; inoculations were made on the same day as heat treatment. The C.S.F. was a pool from seven scrapie-affected goats and was held for 24 hours at 4°C; inoculations were made on the same day as heat treatment.

Dilutions of scrapie goat brain. The starting material used was a thick paste, held at -18°C for four days, made by grinding in a mortar a mixture of four brains from freshly killed scrapie-affected goats. After thawing, the appropriate amount of physiological saline was added to this paste to make a 20 per cent w/v suspension; this was centrifuged at 1,500 r.p.m. for 15 minutes. Doubling dilutions in physiological saline, using separate pipettes for each dilution, were prepared from the supernatant of this suspension. Inoculations were made within two hours of preparing the dilutions.

Determination of beta-globulin phenotypes. The beta-globulin phenotype patterns of goat sera were determined by starch-gel electro-phoresis following the method described by Ashton and McDougall (1958).

RESULTS

Intracerebral Inoculation with Tissues from Clinically Normal Goats

It was reported previously (Pattison and Millson, 1960) that scrapie occured 23 months after inoculation in one of three goats injected intracerebrally with 1·0 ml. of the supernatant of a 10 per cent suspension of frontal cortex obtained from a clinically healthy goat, the history of which was given. The clinical and histological findings were typical of scrapie. Further confirmation of correct diagnosis has now been obtained by the development of scrapie, after an incubation period of some 12 months, in all of three five-month-old castrated male goats (C) subinoculated with brain suspension from this animal.

It was further reported that groups of three goats inoculated intracerebrally with the supernatant of suspensions of brain stem, pituitary gland, adrenal gland, thyroid gland, spleen, pancreas or liver obtained from the same clinically normal animal had remained healthy over a 25-month period of observation. However, it has now to be recorded that one of the three animals inoculated with pancreas suspension developed scrapie 38 months after inoculation. Clinical signs and histological findings were typical of the disease. The remaining animals in this experiment have now been under observation for three years and eight months and no further case of scrapie has occurred (Table 1).

In another experiment a three-month-old, castrated male goat (C) inoculated intracerebrally with 1·0 ml. of a pool of C.S.F. obtained from four clinically healthy goats (C) showed clinical evidence of scrapie some 19 months after inoculation. Symptoms noted were scratching at the base of the neck and over the withers, and slight uncertainty of behaviour; these abnormal signs persisted for about 13 months and then gradually disappeared. The animal is still under observation, some 35 months after inoculation, and has not developed typical symptoms of the disease.

TABLE I

SCRAPIE IN GOATS FOLLOWING INTRACEREBRAL INOCULATION

1·0 ml. amounts of the supernatant of 10 per cent suspensions in physiological saline of various tissues obtained from clinically healthy goats

Experiment No.	*Normal tissue inoculated*	*Origin of tissue*	*No. of goats inoc.*	*Period of observation* months*	*Cases of scrapie: months after inoculation*
1	Whole brain	Heterologous: sheep	12	22	None
2	Frontal cortex	Homologous**	3	44	1 at 23
	Brain stem	,,	,,	,,	None
	Pituitary	,,	,,	,,	,,
	Thyroid	,,	,,	,,	,,
	Adrenal	,,	,,	,,	,,
	Pancreas	,,	,,	,,	1 at 37
	Spleen	,,	,,	,,	None
	Liver	,,	,,	,,	,,
3	Whole brain	,,	2	,,	,,
4	C.S.F.	,,	3	35	1 doubtful at 19
5	Whole brain	,,	2	32	None
6	,, ,,	,,	,,	,,	,,
7	C.S.F.	Autologous	6	21	3 doubtful at 13
	,,	Homologous	3	,,	1 ,, ,, 13
8	Whole brain	Homologous (see Table 2)	,,	20	None
	,, ,,	,,	,,	,,	1 at 17; 1 at 19
	,, ,,	,,	,,	,,	None
9	,, ,,	Homologous	2	15	None
10	Whole brain	Homologous	Two goats inoculated subcutaneously on 30 consecutive days with 10 ml. Under observation 45 months. No scrapie to date.		

* To the time of writing. The animals in Experiment 1 were destroyed for histological examination 22 months after inoculation.

** The animal described by Pattison and Millson (1960).

In order to examine the meaning of the occurrence of scrapie following intracerebral inoculation of tissues from apparently healthy goats, two further experiments have been carried out:—

In the first of these, two castrated male goats and four females (C), approximately three months old, were inoculated with their own cerebrospinal fluid. About 2·0 ml. of C.S.F. were taken from each animal; after storage at -18°C. for two days, this C.S.F. was used for inoculation of 1·0 ml. intracerebrally each animal receiving its own C.S.F. Three other castrated male goats (C) of similar age received 1·0 ml. intracerebrally of a pool of the C.S.F. that remained. Thus.

six goats received autologous and three homologous C.S.F. (Table 1). All were housed in a non-scrapie environment.

Some thirteen months after inoculation, four of the animals (three injected with autologous and one with homologous C.S.F.) showed early evidence of scrapie, being hypersensitive and scratching a little at the base of the neck. In all animals except one these symptoms disappeared within about a month and behaviour became normal again. The exceptional animal had been inoculated with homologous C.S.F.; in this case scratching of the neck, and to a lesser extent over the rump, persisted for five weeks and the animal was very excitable; at this time it was destroyed in order to obtain the brain for subinoculation into four other goats in an attempt to establish a definite diagnosis. Histological examination of the brain failed to confirm definitely that the disease was scrapie, and the subinoculated animals have not been under observation long enough for scrapie to develop.

It remains doubtful, therefore, whether the indefinite signs of abnormality noted in this group of animals may reasonably be regarded as evidence of scrapie associated with the normal C.S.F. inoculated.

In the second experiment brains were obtained from three clinically healthy, castrated male goats (B) about two months old, representing the three beta-globulin phenotypes AA, AB, and BB as described by Ashton and McDougall, (1958). Recipient goats (B) of approximately the same age and also representing the three beta-globulin phenotypes were inoculated as detailed in Table 2.

TABLE 2

SCRAPIE IN GOATS OF KNOWN BETA-GLOBULIN PHENOTYPE

Following intracerebral inoculation on 6.5.59 with 1·0 ml. of the supernatant of 10 per cent brain suspensions in physiological saline from clinically healthy castrated male goats of known beta-globulin phenotype.

Phenotype of donor of normal brain	*Phenotype of recipient inoculated intracerebrally*	*Sex of recipient*	*Occurrence of scrapie* : months after inoculation*
AA	AA	Female	—
	AB	Cast. male	—
	BB	,,	—
AB	AA	Female	17
	AB	Cast. male	—
	BB	Female	19
BB	AA	Cast. male	—
	AB	,,	—
	BB	Female	—
Control inoculum of sterile normal saline	AA	Cast. male	—
	AB	,,	—
	BB	,,	Died, not of scrapie 16 months

* To the time of writing, 20 months after inoculation.

Ashton and McDougall have demonstrated that the beta-globulin phenotype is a genetically inherited characteristic in goats. Brains from normal goats of the different beta-globulin phenotypes were used because of the possibility that the genetical constitution of the donor, and/or the recipient, as indicated by the beta-globulin phenotype, might influence the appearance of disease.

The results of this experiment are given in Table 2, from which it will be seen that clinical scrapie has occurred in two animals up to the time of writing, 20 months after inoculation. Both were inoculated with brain suspension from the donor of AB phenotype; both were females, one of phenotype AA and the other of phenotype BB. The first animal to show evidence of scrapie, 17 months after inoculation, is shown in Fig. 1; the characteristic clinical signs of scrapie developed slowly over some three months. The other animal showed more acute evidence of disease, with scratching high up on the neck, flicking of the tail, shaking of the head and, latterly, impaired vision. It was destroyed two weeks after the appearance of symptoms. Histological examination of the brain showed typical evidence of scrapie with extensive vacuolation of nerve cells in the medulla oblongata. The surviving animals in this experiment remain under observation.

The results to date of the various attempts we have made to produce scrapie by intercerebral inoculation of goats with tissues from clinically healthy goats are summarised in Table 1.

Intracerebral Inoculation with Heat-treated Scrapie Goat C.S.F. or Brain Suspension

Two experiments have been carried out to extend to goats the observation of Wilson (1954) and later workers that the scrapie agent in sheep brain is highly resistant to heat.

In the first experiment, castrated male goats (B) about two months old were inoculated intracerebrally, as detailed in Fig. 3, with 1·0 ml. of pooled C.S.F. from seven scrapie affected goats. It will be seen that only four cases of scrapie occurred, namely in all the three goats inoculated with unheated material and after a longer incubation period, in one of the two animals inoculated with material boiled for 30 minutes. This experiment was discontinued 29 months from the time of inoculation.

In the second experiment, a mixture of the brains of two scrapie-affected goats was ground in a mortar with a little sand and made into a ten per cent suspension in either physiological saline pH 7·4, or phosphate buffered saline pH 7·2. These suspensions were centrifuged at 1,500 r.p.m. for 15 minutes and the supernatant was then removed for heat treatment. Inoculated goats were castrated males (B) about six months old. It had been considered possible that the infectivity of suspensions in physiological saline might differ from that of suspensions in buffered saline; for this reason half the goats received the one inoculum and half the other. In the event there was no detectable difference in infectivity, so that the results have been

Fig. 3

INTRACEREBRAL INOCULATION OF GOATS ON 21.5.58 WITH HEATED OR UNHEATED C.S.F. FROM SCRAPIE-AFFECTED GOATS

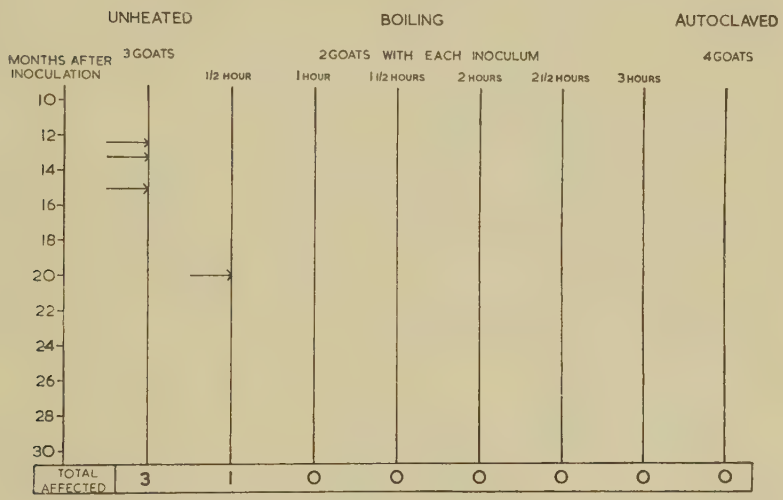
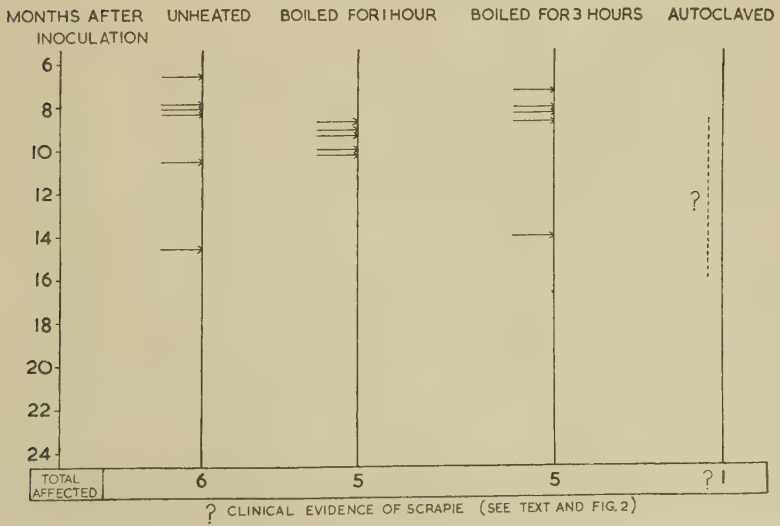


Fig. 4

INTRACEREBRAL INOCULATION OF GOATS ON 24.9.59 WITH HEATED OR UNHEATED SCRAPIE GOAT BRAIN SUSPENSION.

6 GOATS INOC. WITH EACH INOCULUM.



? CLINICAL EVIDENCE OF SCRAPIE (SEE TEXT AND FIG. 2)

combined to give an occurrence of scrapie that is shown in Fig. 4. This experiment has been in progress for some 16 months and the surviving animals remain under observation. It will be noted that one animal inoculated with autoclaved material has been included in the figure as possibly affected with scrapie; this animal is still alive and is shown in Fig. 2. Signs of the disease—uncertainty of movement and scratching at the base of the neck—appeared in this animal some nine months after inoculation, and have progressed only slightly over an observational period of seven months.

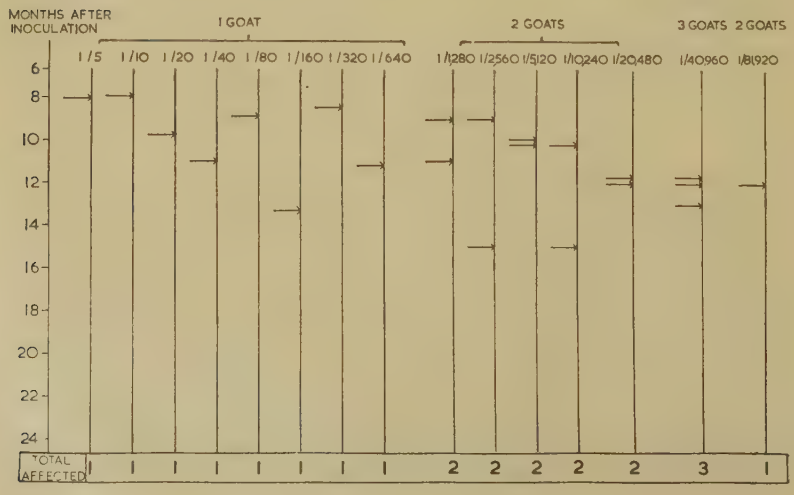
The results of these experiments appear to indicate that while the scrapie agent is highly resistant to heat, continued heating, or autoclaving, reduces the scrapie-producing power. The results in the first experiment contrast with those in the second, and the apparent difference in the degree of the heat resistance in the two experiments may reflect a difference in amount of the agent in the starting materials (C.S.F. or brain suspension) and/or a difference in resistance related to the tissue in which the agent was located.

Intracerebral Inoculations with Dilutions of Scrapie Brain Suspensions

The goats inoculated were castrated males (B) between two and six months of age. The results are given in Fig. 5 from which it will be seen that, over an observation period to date of some 19 months, scrapie has occurred in all the animals except one of the two inoculated with the highest dilution of 1/81,920.

Fig. 5

INTRACEREBRAL INOCULATION OF GOATS ON 13.6.59. WITH VARIOUS DILUTIONS OF SCRAPIE GOAT BRAIN



DISCUSSION

It scarcely requires special comment that further observations are required on the possibility reported here that the scrapie agent may be present in the tissues of some clinically healthy animals. We are aware that the apparent validity of this observation is based on slender evidence. This being so, speculation on the significance of the observations must await the outcome of further experiments.

It will have been noted that on a number of occasions we have encountered transitory abnormalities in inoculated goats that could not be differentiated from the early signs of progressive scrapie. Such observations have now been made too frequently to be dismissed as without significance, and we have been forced to conclude that in certain circumstances, as yet undefined, the experimental disease can reach an early clinical stage to be followed by regression and return to apparent normality. It would be convenient to postulate that such a reaction may be the consequence of a sub-lethal inoculation, but, in the absence of any other test for the activity of the agent, it can only be said that this kind of reaction has only been observed in experiments in which the scrapie-producing activity of the inocula might have been expected to have been reduced.

It does not appear to be likely that this reaction could be a consequence of trauma, for it has not been observed in normal animals following withdrawal of C.S.F. by cisternal puncture, nor in animals inoculated intracerebrally with fluids, e.g.: sterile saline, normal urine or blood, believed not to contain the scrapie agent.

Mackay *et al.* (1960) have commented on apparent differences between scrapie in sheep and scrapie in goats. These comments extend our own observations (Pattison *et al.* 1959) that the disease is not identical in both species. On the basis of these observed differences, Mackay *et al.* have suggested that more knowledge is needed "before the goat can be relied upon as the sole test animal for all purposes." With this we would agree. Nevertheless, looking at reactive differences between the sheep and the goat from a somewhat different angle, we would suggest that to be able to induce the disease in two species is to be able to attack the problem from two angles. Thus, Stamp *et al.* (1959) may well be correct in saying that autoclaving destroys the infectivity of the agent for sheep, but it still remains possible that this treatment may not entirely inactivate the agent as far as the goat is concerned.

During the course of this and earlier work on scrapie we have noted certain similarities between the scrapie agent and the agent associated with experimental allergic encephalomyelitis as defined and described in the authoritative and detailed publication edited by Kies and Alvord (1959). The nature and mode of action of both agents are as yet only imperfectly understood, but certain characteristics of each are fairly well established. Thus, both are highly resistant to heat, to formalin, to freezing and to drying. No circulating antibody directly related to either agent has yet been detected. The

presence of each agent can only be demonstrated by animal inoculation, and in each case it has been suggested that the amount of material inoculated can affect the length of the incubation period. There is a difference among animal species in susceptibility to experimental allergic encephalomyelitis, and this applies also to scrapie (as between sheep and goats). Within the same species, there is a breed and family difference in susceptibility in the case of both diseases.

Stamp, *et al.* (1959) and Mackay, *et al* (1960) have compared scrapie with experimental allergic encephalomyelitis. The former authors have written of scrapie " . . . since tissues other than brain such as lymph glands, spleen and cerebrospinal fluid have now been shown to set up the experimental disease it cannot be merely a form of allergic encephalitis . . . " And the latter have expressed the opinion that "there is good reason to believe that experimental allergic encephalitis and scrapie are quite distinct entities." We would not wish to appear to contradict these conclusions for, from extensive clinical and histopathological studies, it would seem that scrapie and experimental allergic encephalomyelitis are quite unlike pathologically. We feel, however, that the agents associated with both diseases are sufficiently similar to make it worthwhile examining the possibility that similarities in pathogenesis may exist.

CONCLUSIONS

Following our earlier observation that scrapie occurred in a goat inoculated intracerebrally with brain suspension from a clinically healthy goat, the disease has now appeared in one goat inoculated intracerebrally with a suspension of pancreas from the same animal, and in two goats inoculated intracerebrally with a suspension of brain from another clinically healthy goat. Transitory scrapie-like symptoms were observed in five goats inoculated intracerebrally with C.S.F. from clinically healthy goats.

The infectivity of boiled or autoclaved scrapie goat C.S.F. and brain suspension was examined. By intracerebral inoculation of goats the agent was detected in C.S.F. boiled for half-an-hour and in brain suspension boiled for three hours. A scrapie-like syndrome has also developed in one of four goats inoculated intracerebrally with autoclaved brain suspension; this animal is still under observation.

Scrapie occurred in goats inoculated intracerebrally with scrapie goat brain suspension to the maximum dilution tested of 1:81,920.

Certain similarities between the scrapie agent and the agent associated with experimental allergic encephalomyelitis are discussed.

ACKNOWLEDGMENTS

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Fig. 1

Goat showing clinical evidence of scrapie 17 months after intracerebral inoculation with brain suspension from a clinically normal goat. Note scratched area on neck, cocked tail, and "anxious" expression.

Fig. 2

Goat showing clinical evidence of scrapie some 15 months after intracerebral inoculation with autoclaved scrapie brain suspension. Note scratched area on neck.

To face page 358

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Q FEVER AND OVINE ABORTION

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INTRODUCTION

It is now well established that sheep in several parts of the world are infected with *Rickettsia burneti*, the causative organism of Q fever.

Infection in sheep was first shown by Caminopetros (1948) in Greece, and shortly afterwards Lennette, Abinanti, Welsh, Winn, Clark and their colleagues in northern California (Derrick, 1959) provided much valuable information on the spread of infection from sheep to man, the routes of excretion of the rickettsia from infected ewes and the natural history of Q fever in infected flocks. Two reviews (Stoker and Marmion, 1955; Derrick, 1959) may be consulted for details of these findings and also for the geographical distribution of infection.

In Britain Q fever antibody has been found in sheep in Kent (Marmion, Stewart, Richmond, Barber and Stoker, 1954), in two parts of Wales (Report, 1956; Evans, 1956), and in Scotland (Grist, 1956). In addition, the rickettsia itself was isolated from the placenta and wool of two ewes in an infected flock in the Romney Marsh, Kent (Stoker, Brown, Kett, Collings, and Marmion, 1955a).

Relatively little is known about the symptomatology of Q fever in naturally-infected ewes. Although the rickettsia may be present in large numbers in the placenta and birth fluids at parturition and has been found in the foetus and the lamb, there is surprisingly little evidence that it causes abortion. The north Californian workers, for example, found no increased prevalence of abortion in ewes infected naturally or experimentally with *R. burneti* (Abinanti, 1960). However, elsewhere, particularly in Italy, there have been occasional reports of abortion, and sometimes respiratory illness in naturally-infected sheep (Caporale, 1950; Wiesmann 1952). The significance of these reports is difficult to assess because simultaneous infection with other organisms, either known or suspected to cause abortion—organisms of the Salmonella and Brucella groups, *Vibrio fetus*, the virus of enzootic abortion and *Toxoplasma gondii*—was not always excluded.

Since 1958 a survey of the causes of abortion in sheep in Yorkshire has been carried out from the Veterinary Investigation Centre, Leeds. During this survey a flock was found in which a few abortions appeared to be related to a heavy infection with *R. burneti*. This paper describes the findings in the infected flock, the first to be discovered in the northern part of England, and also some serological investigations of sheep elsewhere in Yorkshire which were done to find out whether *R. burneti* was a common, or important cause of abortion in other flocks.

METHODS

All bacteriological examinations were done at the Veterinary Investigation Centre, Leeds. Animal inoculation and serological tests for Q fever were done at the Virus Laboratory, Public Health Laboratory, Leeds. Certain specialised tests were done at the laboratories mentioned below.

Examination of the products of conception for Brucella abortus, *salmonellae and* Vibrio fetus. Smears were made from the stomach contents, and sometimes the organs, of the aborted lambs and from the foetal cotyledons of the placenta. These were stained for the presumptive detection of Brucella organisms by a slight modification of Macchiavello's rickettsial stain. This consists of staining first with dilute carbol-fuchsin, decolourising with three per cent. acetic acid and then counterstaining with methylene blue. The same method, but using one per cent. acetic acid for decolourising, was employed to stain the elementary bodies of enzootic abortion virus.

Foetal cotyledons and portions of the stomach and intestine of the lamb were inoculated into selenite broth(Oxoid). This was incubated at 37°C for 24 hours and subinoculated on to deoxycholate citrate agar (Difco) to detect salmonellae. Stomach contents were also plated on tryptose-infusion blood agar (Difco) and incubated in ten per cent CO_2 at 37°C. for 4 days to isolate Brucella and *V. fetus*.

Sera from the ewes were examined in a tube agglutination test using a standard suspension of *Br. abortus* and incubating at 37°C. in a water-bath for 18 hours.

Examination for Toxoplasma gondii. The method ordinarily employed for attempted isolation of Toxoplasma is described here, although in the particular circumstances of the investigation the inoculated mice were in fact eventually used for the isolation of *R. burneti*. Five gramme amounts of foetal brain, foetal liver and placental cotyledons were ground separately with mortars and pestles with the gradual addition of 15 ml. quantities of saline containing 100 units of penicilliin and 10 microgramms of streptomycin per ml. The suspensions were strained through gauze and all but the largest unground portions of tissue squeezed through the meshes. The strained suspensions were allowed to stand for 2 to 3 hours and the deposits were then inoculated subcutaneously into mice in 1.0 ml. quantities

Serum samples from the ewes were kindly examined for Toxoplasma antibody by Dr. J. K. A. Beverley (Department of Bacteriology, University of Sheffield), using the Sabin-Feldman dye test.

Examination for enzootic abortion virus. Smears were examined for elementary bodies locally, and sera were tested for complement-fixing (CF) antibody to enzootic abortion virus by Dr. J. A. A. Watt at the East Scotland School of Agriculture. Some sera were also tested for psittacosis group CF antibody in the Virus Laboratory, Leeds.

Examination for R. burneti. In general the rickettsia was isolated in guinea-pigs by a method described elsewhere (Andrews and Marmion, 1959). At the beginning of the investigation, when the first two aborting ewes were being examined and the presence of *R. burneti* in the material was first suspected, the mice used for attempted isolation of Toxoplasma were the only inoculated animals which could be examined for Q fever infection: the original samples had been discarded. Some or all of these mice were killed fourteen or more days after inoculation and a ten per

cent. w/v suspension of their livers and spleens was subinoculated into guinea-pigs which were subsequently tested for Q fever CF antibody. The remaining mice were bled three weeks after inoculation and their sera pooled and tested for Q fever CF antibody.

Q fever CF tests with sheep sera were done by the technique of Stoker, Page and Marmion (1955b) using optimal doses—previously determined by 'chessboard' titration with a positive sheep serum—of Phase 1 and Phase 2 antigens prepared from the Christie and Nine Mile strains of *R. burneti* (Stoker and Fiset, 1956). Sera were regarded as positive when they reacted at a dilution of 1/8 or more with either or both of the Q fever antigens and not with a washed suspension of *Rickettsia mooseri* or a suspension of uninfected yolk sac.

Histological methods. Portions of the placental cotyledons were lightly washed and fixed in ten per cent. formol saline. Sections were cut in paraffin, brought to water and placed face-downward on a 1 in 50 dilution of Giemsa stain (G.T. Gurr, London) in phosphate-buffer pH 6.4 for 18 hours at room temperature. They were differentiated with one per cent. acetic acid under microscopic control, passed through acetone, acetone-xylol mixture and xylol and mounted in DePex.

RESULTS

The Infected Flock

The flock, 101 breeding ewes and 3 rams, was on a farm situated in the Ribble Valley on the Yorkshire-Lancashire border. There was also a dairy herd. The sheep were Swaledales, animals having been added to the flock during 1958 by local purchase from three farms. No sheep were purchased during 1959. The animals grazed permanent grassland and were given hay and concentrates during the period before lambing. During tupping the flock was divided into three groups and the three rams moved round every 17 days. Only occasional abortions had occurred in previous years and no examinations had been carried out for possible infective agents.

At the start of the lambing season on March 8th, 1960, one of the ewes (Ewe 1) aborted. The aborted foetus and its placenta were submitted to the Veterinary Investigation Centre for examination. A further single foetus and placenta from Ewe 2 and twin foetuses and placenta from Ewe 3 were received on March 15th and 24th respectively. These three ewes were the only animals in the flock to give birth to dead lambs. Of the remaining ewes, 92 lambed normally, 6 were barren; lambing was concluded by April 20th.

Observations on the Aborted Sheep

Ewe 1. The single foetus together with the placenta had been aborted at about 120 days of gestation. Post-mortem examination of the foetus revealed some small haemorrhagic areas in the liver but no other lesions. Some placental cotyledons were necrotic and others were swollen and haemorrhagic; there was also oedema of the membranes.

Smears from the foetal cotyledons were stained by the modified

Macchiavello technique, decolourising with one per cent acetic acid. This revealed numerous clumps of small red, coccoid organisms morphologically resembling *R. burneti*, lying both intra- and extracellularly. The organisms failed to decolourise even when three per cent. acetic acid was used, and they did not stain with the intensity of bacteria by Gram's method. Smears of the stomach contents were however negative. To confirm the presence of *R. burneti* two groups of mice which had been inoculated, respectively, with placenta and foetal brain suspensions for Toxoplasma isolation were killed 14 days after inoculation. Their spleens were enlarged. Passage of liver and spleen from each group of mice to guinea-pigs showed evidence of Q fever infection (Table I). *Br. abortus* was not isolated from the foetus or placenta but a pure growth of *Vibrio fetus* was isolated from the foetal stomach contents.

TABLE I

ISOLATION OF *Rickettsia burneti*

Products of conception from the three aborting ewes in the Ribblesdale flock

		Titres (reciprocals) of Q fever CF antibody in inoculated guinea pigs nos:-							
		1		*2*		*3*		*4*	
Ewe	*Specimen*	*Pre-‡*	*Post-‡*	*Pre-*	*Post-*	*Pre-*	*Post-*	*Pre-*	*Post-*
1	Placenta[1]	<8	128	<8	128	<8	128	<8	<8
	Foetal brain[1]	<8	<8	<8	128	<8	64	<8	64
2	Foetal stomach contents (diluted)	<8	died	<8	256	..	..	..	..
	Foetal brain[1,2]	<8	<8	<8	<8	<8	<8	<8	<8
3	Placenta	<8	died	<8	512	..	..	..	..
	Milk	<8	<8	<8	256	..	..	..	..
	Foetal stomach contents	<8	128	<8	128	..	..	..	..
	Foetal liver	<8	256	<8	256	..	..	..	..

[1] These specimens were passed through mice before inoculation into guinea pigs.
[2] Pooled sera from the inoculated mice had a Q fever CF antibody titre of 1/32.
‡ Pre- and post-inoculation sera from guinea pigs. .. = not tested.

Serum from the ewe was tested for antibodies against enzootic abortion virus, Toxoplasma, and *Br. abortus* with results which were either negative, or not significant. However, high levels of CF antibody to Phase 1 and Phase 2 Q fever antigens were found in a serum collected sixteen days after the abortion (Table 2).

TABLE 2

SUMMARY OF SEROLOGICAL TESTS

Three aborted ewes in the Ribblesdale flock

Ewe	*Date of abortion*	*Date of bleeding*	*Titre (reciprocal) of complement-fixing and other antibody*					
			Q fever antigen		R. Mooseri (*control*)	*Enzootic abortion*	Brucella abortus *agglutinins*	*Sabin-Feldman dye test*
			Phase 1	*Phase 2*				
1	8th March, 1960	18th March, 1960	..	..	..	<8	<10	58
		24th „ „	128	128	<8	±8	<10	..
2	15th March, 1960	18th March, 1960	..	..	..	<8	<10	..
3	24th March, 1960	24th March, 1960	256	128	<8	<8	<10	42

.. = not tested

Ewe 2. An almost full-term foetus and placenta were received from this ewe. Macroscopic lesions were not seen in the foetus or placenta. Smears from the foetal cotyledons again revealed rickettsia-like organisms and these were also seen in smears of the stomach contents of the foetus.

Salmonellae, *Br. abortus* and *V. fetus* were not isolated from the foetal stomach contents and anaerobic cultures were also negative. Portions of foetal brain had been inoculated into mice and the latter were found to have Q fever antibody when they were bled three and half weeks after inoculation. However *R. burneti* was not isolated by the subinoculation into guinea-pigs of liver and spleen suspension collected from these mice at the time of bleeding (Table 1). Burnet and Freeman (1938) found that there was difficulty in passing *R. burneti* serially in mice when the liver and spleen were harvested on or after the fourteenth day, presumably because the rickettsiae were neutralised by newly-formed antibody. This effect is probably also responsible for the failure to infect all the guinea-pigs by subinoculating liver and spleen from the mice inoculated with placenta or foetal brain from Ewe 1 (Table 1). Hence the apparent contradiction between the large numbers of organisms seen in direct smears from these specimens and the relatively poor end result in infecting guinea-pigs.

Inoculation of undiluted foetal stomach contents (Ewe 2) into guinea-pigs killed the animals, but *R. burneti* was isolated when the stomach contents were diluted with bacteriological culture medium and then inoculated into guinea-pigs, (Table 1). Unfortunately a serum specimen from this ewe was not available for Q fever antibody tests but one was tested for brucella antibodies and for complement-fixing antibodies to enzootic abortion virus with negative results (Table 2).

Ewe 3. One of the aborted twins was approximately 140 days old and the other, unsuitable for examination because of putrefaction, was about 90 days old.

No macroscopic lesions were present in the older foetus but the placental membranes were discoloured and thickened and the cotyledons were necrotic. Large numbers of rickettsia-like organisms were again seen in smears from the foetal cotyledons and from the foetal liver, but those from the stomach contents were negative. Salmonellae were not isolated from the foetal cotyledons or the stomach contents. *Br. abortus, V. fetus* and anaerobic bacteria were not isolated from the stomach contents. Suspensions of foetal stomach contents, foetal liver, placental cotyledons and also milk from the ewe were inoculated into guinea-pigs and *R. burneti* was isolated from each specimen (Table 1). The milk was cultured for *Br. abortus* with negative results. Serological examination of the ewe gave negative results in tests for Brucella, enzootic abortion virus and Toxoplasma antibodies but high titres of Q fever CF antibody were found (Table 2).

Histological Observations on the Aborted Placentas

The foetal cotyledons from Ewes 1 and 2 were examined and these showed some necrosis and infiltration with mononuclear and eosinophil cells but of a lesser degree than that described in viral enzootic abortion (Stamp, McEwen, Watt and Nisbet, 1950). There were also cells—presumably those of the surface epithelium of the chorion and foetal cotyledons—containing microcolonies of rickettsia-like organisms (Fig. 1). These colonised cells closely resembled the cells packed with elementary bodies found in viral enzootic abortion (Stamp *et al.*, 1950) but, as already described, this infection had been excluded by serological tests on the aborting, and other, ewes in the flock. Rickettsia-like organisms, but surprisingly few bacteria, were seen lying free in the space between the foetal cotyledons.

Observations on Other Sheep in the Flock

On April 4th the flock was visited and 73 ewes which had lambed normally were bled; several ewes had still to lamb. As fifteen (20.5 per cent.) of the 73 ewes sampled had antibody to Phase 2 CF antigen, some at quite high titres (Table 3), infection was widespread in the flock. Only two ewes had antibody to Phase 1 CF antigen: both at a titre of 1/32.

Examination of Cattle on the Farm

At the time of the first visit to the farm the farmer suggested that the abortions in the sheep might be related to some which had occurred in the dairy herd. Five cows had aborted during 1959 and two during 1960. Blood samples were taken from two of these animals

TABLE 3

DISTRIBUTION OF COMPLEMENT-FIXING (CF) ANTIBODY

Titres to Q fever Phase 2 antigen (Nine Mile strain) in (a) normally lambing ewes on the Ribblesdale farm, (b) in aborting ewes in 60 Yorkshire flocks and (c) in normally lambing ewes in the same flocks.

Sera from	Distribution of CF antibody titres (reciprocals) to Q fever, Phase 2 antigen								Total
	<8	8	16	32	64	128	256	512 or more	
Ribblesdale flock									
(a) Normal ewes	58	3	7	1	1	2	0	1	73* (20.5)
General Survey									
(b) Aborting ewes	206	4	6	4	0	0	0	0	220 (6.4)
(c) Normal ewes	135	2	4	2	1	0	0	0	144 (6.3)

( ) Sheep (percentage) in subgroup with antibody titres of 1/8 or more

* All sera were negative with psittacosis group CF antigen

which had aborted 8 months and 5 months previously. Agglutination tests for Brucella antibody and complement-fixation tests for Q fever antibody were negative. Milk samples from both cows were negative on culture for *Br. abortus*.

On May 3rd, another cow aborted and the foetus was received for examination together with milk samples from this animal and from the two which had aborted earlier in 1960. Brucella and rickettsia-like organisms were not seen in smears from the foetal stomach contents and cultures incubated aerobically, anaerobically and in 10 per cent. CO_2 were negative. The stomach contents and the three specimens of milk were inoculated into guinea-pigs but *R. burneti* was not isolated.

Survey of Normal and Aborting Sheep in Yorkshire

Sixty flocks in all three Ridings, but mainly in the West and East, were investigated. In each flock blood samples were collected from ewes which had aborted and from a smaller number of ewes which had lambed normally. These sera were tested for Q fever antibody with the results which are shown in Table 3. Fourteen (6.4 per cent.) of the 220 aborting ewes and nine (6.3 per cent.) of the 144 which had lambed normally had antibody to Q fever Phase 2 CF antigen at a titre of 1/8 or more. Only two of these reactors also had antibody to Phase 1 CF antigen—both at dilutions of 1/16—so that the titres of both Phase 1 and 2 antibody were well below those observed in the aborted ewes (Tables 2 and 3). Infection was not spread uniformly through the 60 flocks, only 12 being positive. Even when allowance was made for this in analysing the figures, and also for infection with other organisms known to cause abortion in some of the flocks, there was still no evidence that Q fever was a general and important cause of abortion in Yorkshire sheep.

DISCUSSION

The findings in the Ribblesdale flock are of interest in several ways. The isolation of *R. burneti* from the placentas and the ewe's milk, and from foetal brain, liver and stomach contents provides a useful confirmation of the single, earlier report on the presence of the organism in sheep in this country. The intensity of infection in the Ribblesdale flock was impressive—20 per cent. of the animals had antibody, and organisms resembling *R. burneti* were seen in large numbers in smears or sections of the products of conception from the aborting ewes. This contrasts with the generally low intensity of infection observed in the Romney Marsh flocks (Marmion *et al.*, 1954; Stoker *et al.*, 1955a), although in one exceptional flock 33 per cent. of the animals did have antibody. The situation more closely resembles that described in north Californian flocks (Welsh, Lennette, Abinanti and Winn, 1951). The presence in the sera of the aborting ewes of CF antibody to the Phase 1 antigen of *R. burneti* is also of interest. Such antibody was not found at high titre in the other sheep

tested, nor did Stoker and Fiset (1956), who examined sera from 111 infected sheep, find Phase 1 antibody above a titre of 1/10. Its formation in the aborted ewes was probably a response to the intense antigenic stimulus provided by a particularly heavy infection. An analogy exists with the development of Phase 1 CF antibody by human beings with chronic Q fever endocarditis and its absence in the short-term, uncomplicated human case of Q fever (Andrews and Marmion, 1959).

Although *V. fetus* was found in one of the three ewes, no known cause of abortion was found in the other two—the low titres of Toxoplasma antibody are not considered to be of significance. Although the latter organism has been associated with ovine abortion in England (Beverley and Watson, 1959), antibody at a titre within the lower positive range of the dye test is commonly found in the sera of sheep without clinical symptoms. It seems reasonable to conclude that, in the unusual circumstances of the very heavy infection, the abortions were caused by *R. burneti*. It is clear from the survey of sheep at large, however, that Q fever is not a common or important cause of abortion. The reason for this innocuousness is obscure, but it has been suggested that extensive multiplication of *R. burneti* in the ovine placenta is delayed until the very last stages of gestation (Abinanti, 1957). It may be pertinent that although large numbers of rickettsia-like organisms were seen in the placentas from the three aborted ewes the organisms appeared to occupy a superficial position and to evoke relatively little cellular or necrotic response. It may be noted, however, that in another ruminant—the goat—at least one widespread outbreak of abortion attributed to *R. burneti* has been recorded (Kilchsperger and Wiesmann, 1949).

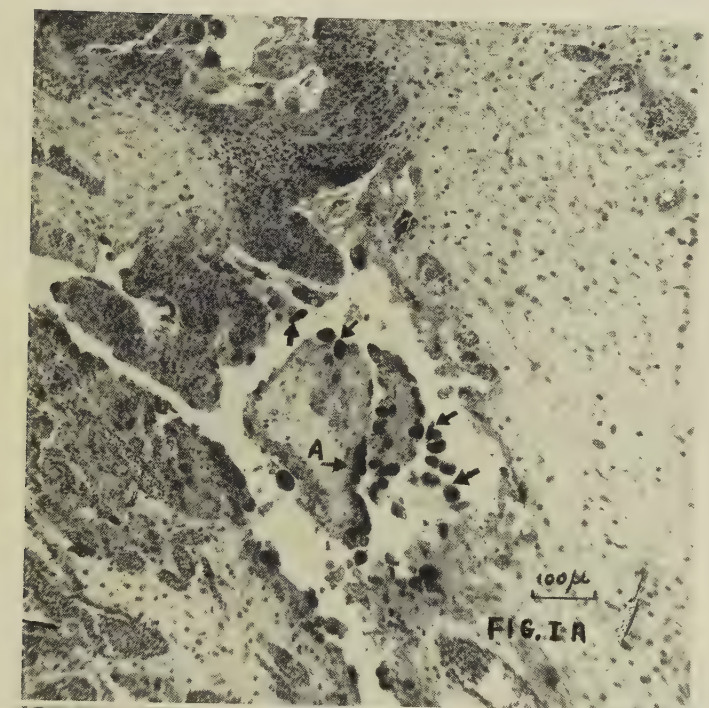
In spite of the high incidence of infection in the Ribblesdale flock there was no evidence of associated disease in the cattle or farm workers. However a technician in the veterinary laboratory who processed the placentas for mouse inoculation did develop Q fever. It is clear that the examination of specimens from aborted sheep, cattle and goats represents a hazard for workers in veterinary laboratories.

CONCLUSIONS

During an investigation of the causes of ovine abortion in Yorkshire three abortions were observed in a flock in the Ribble valley. Rickettsia-like organisms were seen in smears made from the aborted placentas, foetal liver and stomach contents and also in large numbers in histological sections of the placental cotyledons.

Rickettsia burneti was isolated from the products of conception—placenta, foetal liver, brain and stomach contents—of the three ewes and also from the milk of one of them. The aborting ewes had high levels of Q fever complement-fixing antibody and over twenty per cent. of the remaining ewes in the flock also had Q fever antibody.

Although *Vibrio fetus* was isolated from one of the three aborted foetuses, no cause was found in the other two ewes or their foetuses.



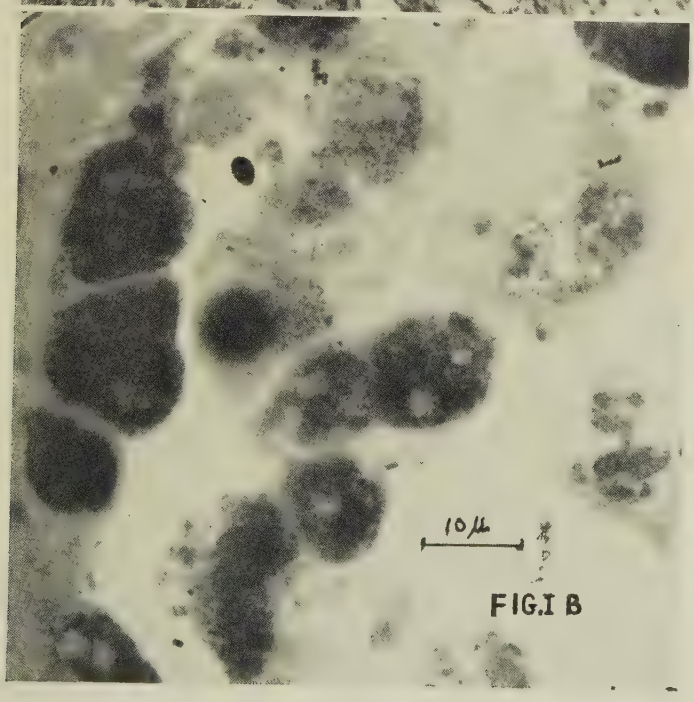


Fig. 1 Giemsa-stained section of foetal cotyledon from Ewe 1. (a) low power view showing slight cellular infiltration and cells with microcolonies of rickettsia-like organisms (marked by arrows) and (b) high power view of microcolonies in the area marked by 'A' and an arrow in the photograph (a).

To face page 368

These findings confirm the single, earlier report of the isolation of *Rickettsia burneti* from British sheep and suggest that abortion may accompany a very heavy Q fever infection.

A survey of sixty other flocks with aborting sheep revealed serological evidence of infection in twelve. There was, however, no significant difference in the incidence of Q fever antibody in aborting and non-aborting ewes in the infected flocks and it is concluded that Q fever is not a common cause of ovine abortion.

A laboratory technician developed Q fever after unsuspectingly handling an aborted foetus and placenta, and attention is drawn to this hazard in veterinary laboratories.

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HAEMANGIOENDOTHELIOMAS IN POULTRY

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INTRODUCTION

Small bleeding skin lesions are not uncommon in fowls. They may be localized in any part of the body, but they appear to occur most frequently on the hock joint, especially on the point of the hock and on the volar side of the carpal joint. The lesions have often been interpreted as signs of cannibalism, but microscopic examination has shown that they were vascular tumours.

Vascular tumours appear to be more rare in mammals than in fowls. Adamek (1934) found in his survey of the relevant literature that the frequency of vascular tumours in relation to other tumours was approximately 0.4 per cent. in horses and dogs and about 2 per cent. in fowls. Olson and Bullis (1942) observed 5 haemangiomas among 384 tumours in chickens (1.3 per cent.). Nieberle and Cohrs (1952) reported that haemangiomas are most common in fowls and that usually it is a question of primary multiplicity with lesions arising in several organs simultaneously. Schürmann (1928) described multiple haemangiomas in several tissues and organs of a Leghorn chicken four to five months old. On microscopic examination these tumours exhibited all the transitional stages from solid collections of angioblasts to large blood-filled cavities lined with endothelium. The authors termed them cavernous haemangiomas. Sal (1930) reported vascular tumours in five Leghorn fowls between six months and one year of age and belonging to the same flock. The tumours were situated in heart, liver, lungs, skin and eye-lid. In the skin there were blood-filled cavities surrounded by a capillary network in the deeper layer of the corium. The tumours were termed haemangiomas and some of them had a diffuse immature connective tissue stroma.

In his survey of vascular tumours in animals, Adamek (1934) stated that in fowls these are usually localized in the skin and from a clinical point of view they manifest themselves as haemorrhages of the skin. They vary in size from a pin's head to a fist. According to the same author multiple haemangiomas and haemangioendotheliomas frequently occur in fowls and in several flocks the outbreaks assumed enzootic proportions. In a large number of tumours it was possible to observe along with vascular tissue simultaneous neoplasia of dense connective tissue, adipose tissue, and smooth musculature. Olson and Bullis (1942) described vascular tumours in the liver and kidneys of five hens aged 22 to 52 weeks. In three birds the tumours were cavernous haemangiomas and in two, capillary haemangiomas.

Monlux and Delaplane (1952) investigated haemangiomas in the skin of eight young White Leghorns from three flocks but originating from a common parent stock. The size of the tumours varied from scarcely visible reddish-blue spots to round, bulging purplish red nodes about one cm. in diameter. The larger tumours projected above the surface of the skin and were consequently more exposed to traumata which could give rise to bleeding. Histologically it was observed that the tumours developed

from small capillary foci in the subcutis to large masses of winding capillaries. Some of the tumours contained packed endothelial cells arranged in filaments. Determination of bleeding time, clotting time, Hb-values, total cell counts and cell morphology showed no abnormalities.

Darcel and Franks (1953) described skin tumours on the neck in 13 chickens aged 3 to 5 weeks. The skin was thickened and in some cases it was ulcerated. In the corium, groups of cells resembling endothelial cells were observed and also cavities that often contained blood. Rigdon (1954) found haemangiomas in 11 chickens; most of these were in the skin of the head and neck and the rest were in the musculature. The majority of the tumours were multiple and many of them recurred during a period of 2 to 6 months.

RESULTS

Macroscopic Appearances

Out of 21,800 poultry autopsied at the State Veterinary Medical Institute, 1953 to 1959, 433 had tumours (leukosis and neurolymphomatosis not included) of which 27 were vascular tumours (Table 1). Among broilers of one flock, 15 tumours were observed 13 of these being vascular tumours.

TABLE 1

TUMOURS IN POULTRY 1953-59

	Vascular tumours	*Leukosis*			*Neuro-lymphoma-tosis*	*Other tumours*	*Other cases*	
		Lymphoid	*Myeloid*	*Erythro-blastic*				
1953	3	801	12	1	389	72	2,172	
1954	6	745	17	–	448	61	2,277	
1955	3	883	16	1	645	59	2,851	
1956	3	633	17	–	334	61	1,963	
1957	4	296	6	–	145	53	1,524	
1958	5	491	15	–	335	58	1,816	
1959	3	494	12	–	391	42	1,658	
	27	4,343	95	2	2,687	406	14,261	Total 21,821

Twenty birds with vascular tumours were examined more closely. Of these, 14 were cross-bred broilers from the same flock and from six weeks to four months old. The other breeds represented were White Leghorn and Plymouth Rock. There were 11 birds with multiple tumours and in 13 the tumours were located either wholly or partially in the skin. Table 2 shows the sites of the tumours. Six of the chickens had tumours in the subcutis on the metatarsus; two of

TABLE 2

LOCALIZATION OF THE VASCULAR TUMOURS

Autopsy No. 1960	*Breed*	*Sex*	*Weight kg*	*Skin*	*Liver*	*Lung*	*Spleen*	*Kidney*	*Heart*	*Oviduct*	*Subperitoneal fat*	*Musculature*	*Synovial sheath*
1489	Br	♀	1.65	+ (leg)								+	
1627	Br	♀	2.20	+ (leg)					+				
2057	Br	♀	2.55										+
2259	Br	♂	1.88										
2738	Br	♂	1.87	+ (head)		+	+					+	
3253	Br	♀	0.58	+ (leg)	+	+		+					
3325	Br	♀	0.50	+ (wing)									
3934	Br	♀	0.80		+	+						+	
4148	Br	♀	1.20		+	+							
4149	Br	♂	1.32	+ (leg)		+		+					
4593	Br	♀	0.67	+ (leg, head)									
5149	Br	♀	1.15	+ (neck)									
5443	Br	♂	1.90		+		+					+	
5542	Br	♂	0.11	+ (leg)									
1739	WL	♂	0.37	+ (neck)									
2037	WPR	♀	0.06*	+ (wing)									
2087		♀	1.66		+								
4876		♀	1.85		+					+		+	
5487	WL	♀	1.45	+ (wing)	+		+						
6292	WL	♀	1.75	+ (head)		+					+		

Br = Crossbread broilers from the same flock.
WL = White Leghorn
WPR = White Plymouth Rock
} These birds were from different flocks.
* = 11 days old.

these tumours were localized on the plantar surface and somewhat medially on the hock joint. Here small wounds were detected, covered with dried blood-clots surrounded by a diffuse grey-violet area (Fig. 1). The tumours contained much blood. The tumours in the skin covered by feathers, in the musculature and the synovial sheaths varied from a pea to a walnut in size; they were relatively well defined and sanguineous. The skin tumours frequently bled from surface lesions and could cause death by exsanguination. In a number of these birds blood was found in the crop. In three of the cases the tumours were localized in the volar surface of the carpal joint. In the internal organs tumours were observed which varied in size from one mm. to one cm. in diameter and were well defined, dark blue and often projected above the surface. They were frequently multiple (Fig. 2). In two birds the tumours in the liver had ruptured with haemorrhage to the abdominal cavity; and in one bird with vascular tumours in the apex of the heart death had been caused by cardiac tamponade.

Microscopic Appearances

Histological examination showed not only wide variations in the appearance of different tumours but also in individual tumours especially in those in the skin. However, three of these tumours in the skin of the wings and neck (one of these appeared in a chicken 11 days old) had quite a uniform appearance. When the lesions invaded the subcutis, tissue of a sarcomatous nature developed and was widely spread: in places this tissue had even infiltrated the dermis and the epidermis, and here the skin was often ulcerated (Fig. 4). The tumour tissue consisted of tightly-packed large polygonal or oval cells with abundant cytoplasm, relatively large polymorphous nuclei and a loose chromatin network (Fig. 5). There was a network of fine silver-positive fibres. In some areas a few collagen fibres were also found. Here and there in the tumorous tissue cavities tended to differentiate; in a number of cases these were strongly dilatated and filled with blood (Fig. 6). This type of tumour may be designated as very immature and only slightly differentiated.

Tumours located on the leg were distinguished morphologically from those in the parts of the skin covered with feathers by their more pronounced tendency to form collagen. In these tumours islands were observed consisting of immature cells with abundant cytoplasm surrounded by fine collagenous fibres and displaying a tendency to form slit-like vascular spaces. Around these islands were dense bands of collagenous tissue. These streaks often contained drops of fat and haemosiderin. In the other skin tumours various transitional stages were observed, ranging from the above-mentioned immature areas with an abundance of cells and having a sarcomatous character, to densely collagenous areas (Fig. 7).

The tumours in the internal organs were generally composed of loose sarcomatous tissue, with immature cells resembling those found

in the skin tumours. An abundance of silver-positive fibres was found between the tumorous cells. For the most part, collagen formations were less pronounced than in the skin tumours. Here and there a tendency to form vascular cavities was noted; often these were cavernous and sometimes were surrounded by a connective tissue capsule. The interior surface of the capsule had an endothelial lining. In the liver small groups of liver cells were occasionally enclosed. In some of the tumours accumulations of blood resembled haematomas, and were surrounded by a connective-tissue capsule without an endothelial internal lining. In the areas around such blood-filled cavities in the liver, the cells had frequently been compressed. In individual cases, in more developed types of tumours in the skin and the internal organs, dilatated cavities were sometimes observed, which, besides blood, also contained bands of tumour tissue with a varying collagen content. In the liver tumours in two birds focal accumulations of elongated, often confluent, ducts were found. The ducts were lined with simple cubical or cylindrical epithelium resembling bile-duct epithelium. The lumina often contained eosinophil masses (Fig. 8).

In one bird, the tumours in the lung were rich in collagen, with here and there more immature loose areas with a large number of cells and a network of fine vascular spaces. In the immediate proximity of the larger blood-filled cavities fine spaces were observed which also contained blood. As the large cavernous cavities became even larger, the pressure on the surrounding areas increased and the small spaces were compressed. Haemosiderin pigment was noted in the capsule. In another bird widespread proliferation of vascular endothelium in both large and small vessels was observed in the tumours of the lungs. Immature endothelial cells bulged into the lumen, and diffuse infiltration of tumour cells into the walls of a large vessel could be seen. Otherwise the picture displayed the ordinary features, with areas that manifested higher and lower degrees of differentiation, but the amount of collagen present was slight.

DISCUSSION

The tumours displayed various degrees of differentiation. Variations were frequently observed in the same tumour and it was possible to follow the development from an immature, initial cell-rich stage. In some birds the lesions had bled severely. The tumours do not appear to metastasize or to cause tumorous cachexia. They do not grow to any appreciable extent, but they are frequently multiple.

The condition can be regarded as a multiple, relatively benign tumour, which in some flocks occurred mainly in very young birds.

It is assumed that the tumours are congenital or are acquired at a very early age. If they are congenital, which this material does not definitely show, the cause may be an hereditary developmental disturbance or an infection in ovo. Willis (1953) and v. Albertini (1955) consider that many vascular tumours are vascular malformations. On the other hand, transmissions of virus-induced neoplastic

tissue has been observed in fowls with leukosis (Burmester, Gentry and Waters, 1955). It has also been shown that, under certain experimental conditions, leukosis virus may give rise to tumours of an angiomatous character (Oberling, 1954). In some of the birds described here, a very immature sarcomatous stroma was observed and in the tumours in the liver there was a proliferation of bile-duct epithelium. Such findings in a vascular tumour may be interpreted as indicating a teratoid tendency. In spite of this, however, they need not be taken as unquestionably supporting the hypothesis of malformation. In this connection attention may be drawn to the experiments of Thorell (1958). The inoculation of one-day-old chicks with leukemia virus did not as a rule induce leukosis, but renal tumours of a teratoid type developed. The question of the cause of vascular tumours must naturally be left open until further studies have been made, especially with regard to transmission experiments.

The most usual localization is the skin, possibly because only slightly differentiated mesenchymal tissue persists here and this tissue can readily give rise to the formation of tumours (Schürmann, 1928; v. Albertini, 1955). Layers of dense connective tissue are observed in the subcutis of the leg. One layer is situated just near the dermis and the other deeper and adjacent to the synovial sheaths and the periosteum. In between these layers there is a more open region containing a large number of capillaries. Between the capillaries immature connective tissue, rich in cells resembling fibroblasts, is found (Fig. 3). The very immature appearance of this tissue may be due to the fact that the differentiation of the mesenchymal structure is still in progress, a possible explanation for the relatively high incidence of tumours in this site.

The tumours are biologically benign although their histological appearance is malignant. The occurrence of immature endothelial cells has most often overshadowed the appearance of capillaries. The skin tumours have displayed a strong tendency to form collagen, which apparently began at an early stage and then formed a link in the devlopment—or regression—of the tumours. This has resulted in a partial sclerosis and consolidation. In individual cases rupture and bleeding have given rise to large blood-filled cavities. It would seem most appropriate to designate these tumours as benign sclerotic haemangioendotheliomas.

The tumours in the internal organs have retained to a great extent an immature aspect with slight differentiation of collagen fibres. Apparently they should also be termed benign haemangioendotheliomas which, in some instances, exhibit a tendency to form cavernous cavities.

CONCLUSIONS

Out of 433 tumours in chickens, 27 were of vascular origin. Twenty tumour-bearing birds were investigated in detail of which 14 were broilers from one flock. There were 11 birds with multiple

tumours in the skin, musculature and internal organs. In seven birds the tumours were limited to the skin, whereas in six birds skin tumours were concomitant with tumours in the internal organs. Both the skin tumours and the visceral tumours were relatively small, usually varying from one mm. to one cm. in diameter and they had a propensity for haemorrhage.

The degree of differentiation varied not only in the different birds but also within the individual tumours. In areas with very low differentiation a sarcomatous tissue with pale, polygonal cells with abundant cytoplasm was found together with a large number of fine silver-positive fibres. At higher degrees of differentiation, fine spaces resembling capillaries or large cavernous blood-filled cavities had developed. In some parts of the stroma, highly differentiated collagen fibres were found, especially in the skin tumours. In two liver tumours a simultaneous proliferation of the bile ducts was observed.

The tumours did not appear to increase appreciably or to metastatise. They occurred both as single and as multiple tumours and were designated as haemangioendotheliomas.

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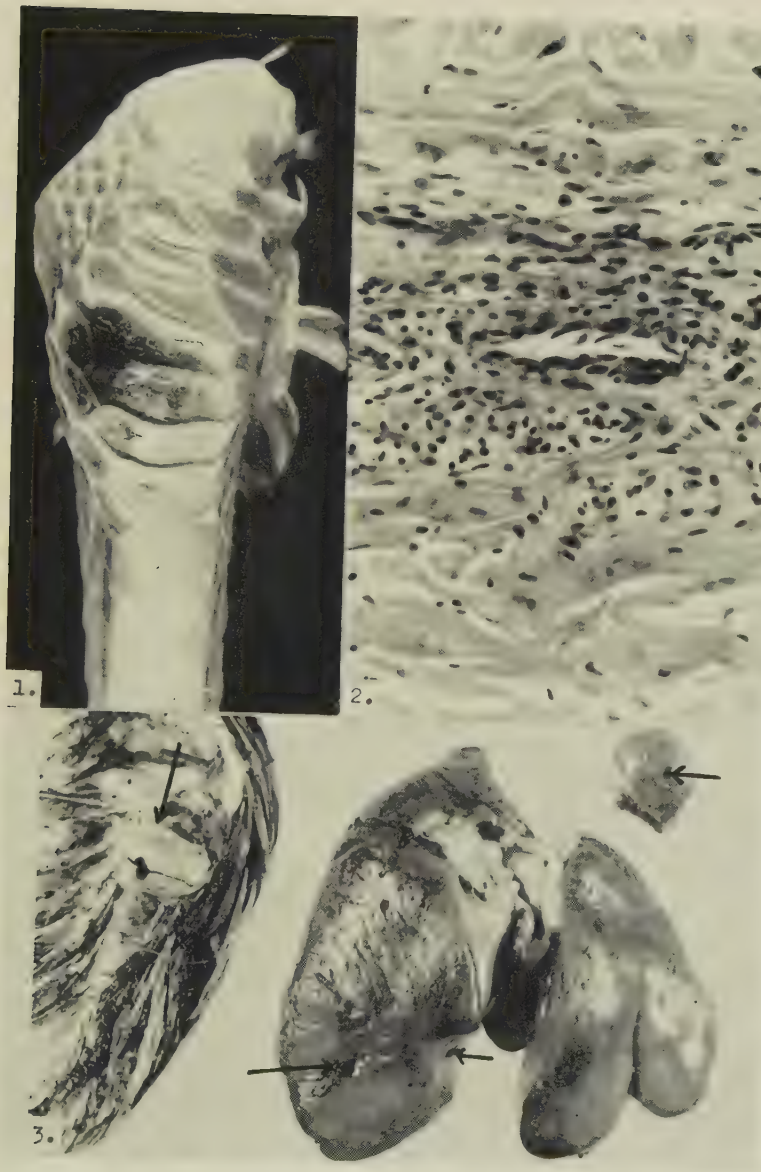

Fig. 1. o 1627/60. Broiler. Vascular tumour on point of the hock.
Fig. 2. o 3552/60. Normal appearance of the subcutis on the metatarsus. A relatively loose area, rich in capillaries, lies between two dense layers of connective tissue. Haematoxylin and eosin. x 400.
Fig. 3. o 5487/60. White Leghorn. Tumour, about 1.5 cm. in diameter, on the under surface of the left wing. The tumour bled from a small wound. Scattered tumours, varying in size from 1 mm. to 5 mm. in diameter, in the liver and spleen. Haemorrhage into the abdominal cavity from the tumour situated on the right hepatic lobe.

To face page 376

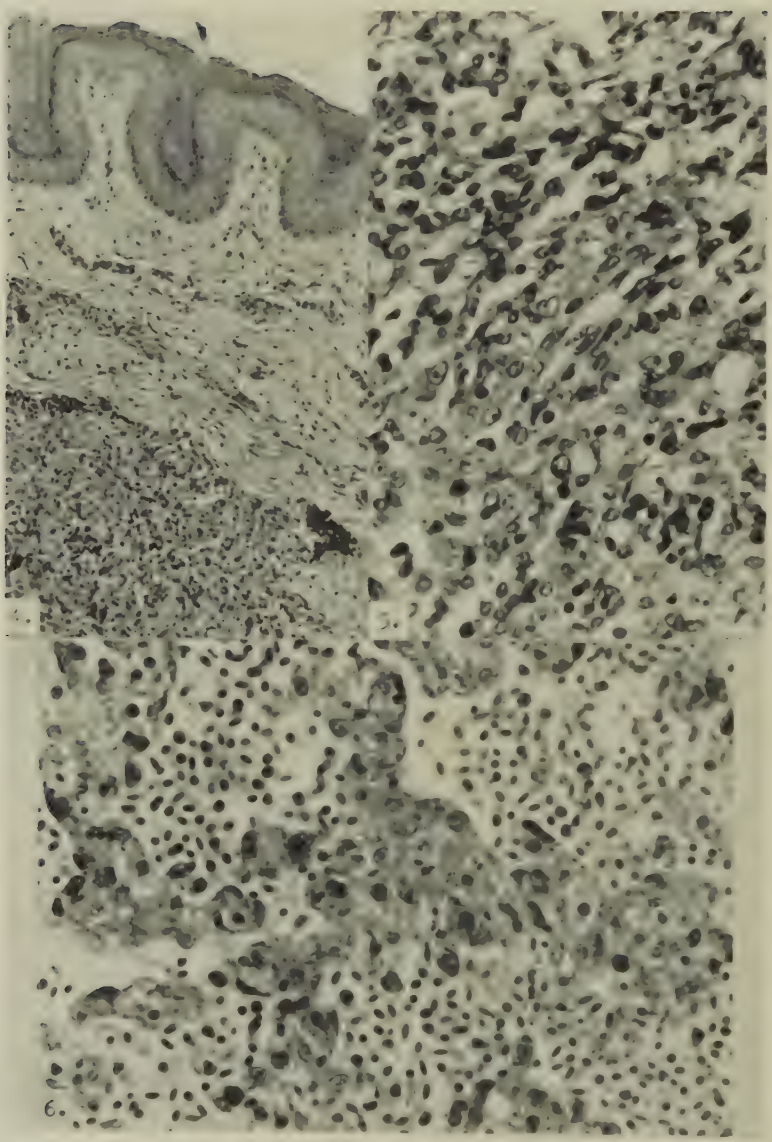

Fig. 4. o 1739/60. White Leghorn. Skin tumour in subcutis on the neck. The tumour is predominantly solid and sarcomatous. Haematoxylin and eosin. x 120.

Fig. 5. Detail of the previous tumour. Tendency to form vascular spaces with differentiation of collagenous fibres. Haematoxylin and eosin. x 400.

Fig. 6. o 2037/60. White Plymouth Rock, 11 days old, Skin tumour on the wing. Plump polygonal cells form bands and islands surrounded by blood. Haematoxylin and eosin. x 400.

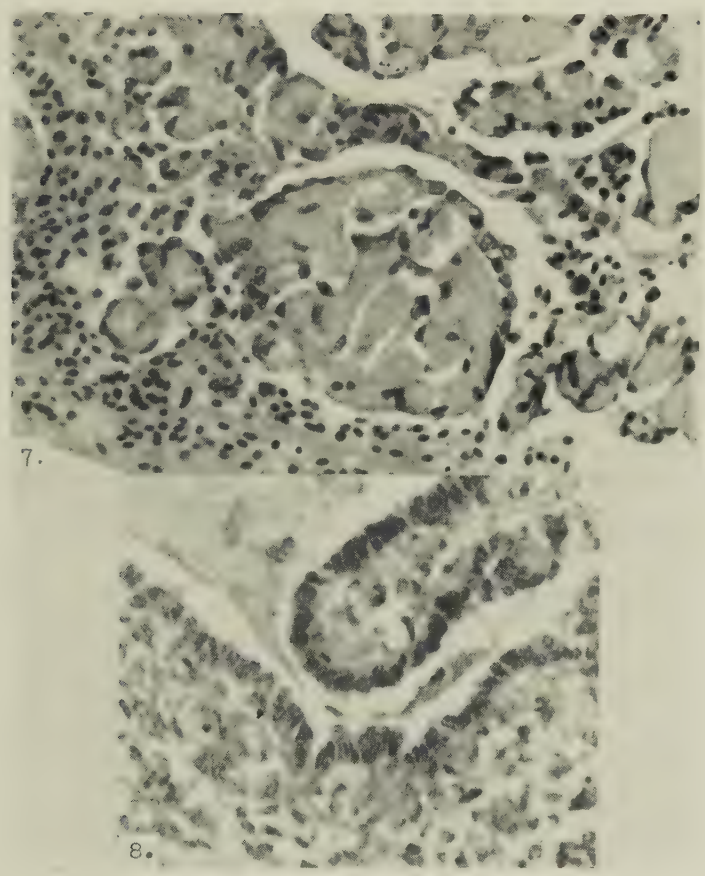

Fig. 7. o 2738/60. Broiler. Tumour in the skin of the head. Differentiation of large and small blood-filled vascular spaces lined with immature endothelial cells and containing collagenous fibres in the walls. Haematoxylin and eosin. x 400.

Fig. 8. o 2087/60. White Leghorn. Tumour in the liver. The tumour has a sarcomatous character and is only slightly differentiated. The bile-ducts in the tumour are proliferated. Haematoxylin and eosin. x 400.

NECROTIC ENTERITIS IN THE FOWL (*GALLUS GALLUS DOMESTICUS*)

I. Histopathology of the disease and isolation of a strain of *Clostridium welchii*

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INTRODUCTION

A study was made of an enterotoxaemia of young cockerels apparently caused by *Clostridium welchii*. The condition was observed in a large group of cockerels which had recently been moved to new premises, and numbers of the birds commenced to die soon after. The investigations were carried out to determine the nature of the disease and its predisposing causes as part of a study of enteritis of the chicken, and to obtain information which could be relevant to the condition known as 'enteritis necroticans' or 'Darmbrand' occurring in man as described by Beckermann and Laas (1946) and Jeckeln (1947) with which it appeared to have many similarities.

This paper presents the histopathological changes observed in the natural disease, and records the isolation of a strain of *Cl. welchii* and a mouse lethal toxin from the lumen of the small intestine of the affected birds.

MATERIALS AND METHODS

Post-mortem examinations of the birds dying of the disease were carried out as soon as possible after death, often within 6 hours. Many of the birds whose tissues were used for histological examination were killed in extremis by Nembutal injected intravenously and a post-mortem examination carried out immediately. Small pieces of all organs and sections at different levels of the intestine, whether affected or not, were taken from every carcase examined and fixed in 10 per cent formal saline pH 7·0, Lendrum's formal-sublimate solution or Carnoy. The tissues were embedded in paraffin, and staining techniques were performed as described by Pearse (1953) except for Danielli's tetrazo reaction for protein containing tyrosine, histidine or tryptophane, for which the method of Dixon (1953) was used. The ribonuclease and deoxyribonuclease used for extraction of the sections were obtained from Light's Chemical Co. Smears made of contents from the lumen of affected portions of the intestine were stained by aqueous methylene blue, Gram's and Macchiavello's techniques.

Material for bacteriological examination was taken from affected and normal areas of intestine, liver, bile, spleen, heart blood, kidneys and bone marrow and was plated out on sheep blood agar under aerobic and anaerobic conditions. About 250 mg. portions of these organs from some of the birds were ground in Griffith's tubes and used to inoculate

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beef infusion broth media enriched by 20 per cent chicken serum pH 7·6 and tubes of Robertson's meat media. The contents from the lumen of normal and affected areas of the small intestine were diluted 1/4 v/v in phosphate buffered saline, ground in a Griffith's tube, centrifuged for 1 hour at 3000 r.p.m. and the middle layer removed with a pasteur pipette. This extract was filtered through 0·4 μ gradocol membranes and injected in 0·2 ml. volumes intravenously into mice. Neutralization tests of the mouse lethal toxin obtained in this extract were carried out on 0·4 ml. of extract with an equal volume of antisera at 37°C for 30 mins, and 0·2 ml. of the toxin-antitoxin mixture was injected into each of three mice. When a sufficient volume of extract was obtained, all 4 antisera were used for neutralization tests on material from each bird. The antisera were stated to have the following potencies:— Anti type A contained 150 units anti α toxin/ml.; anti-B, 400 units anti β and 25 units anti ϵ/ml.; anti-C 730 units anti β/ml.; anti-D 370 units anti ϵ and 25 units anti α. A pool of the toxic extract obtained from 5 birds was injected intravenously in 1·0 ml. volumes into 4 young White Leghorn cockerels of an average weight of 750 g.

Extracts of the faeces, contents of the small intestine and body organs were inoculated into tubes of chick embryo monolayer cell cultures prepared after the method of Bodian (1956).

Venous blood was taken into a mixture of dry ammonium oxalate and potassium oxalate in tubes containing 6 mg. and 4 mg. respectively, or into dry heparin and gently agitated. Any samples with clots were discarded. Two samples from each bird were centrifuged in Wintrobe haematocrit tubes for 30 min. at 3,000 r.p.m. After the blood volume readings had been made, the top pale layer was removed with a pasteur pipette, resuspended in 10 per cent chicken plasma in phosphate buffered saline, and smears were made for microscopical examination and stained with Leishmann.

RESULTS

Clinical Findings

The disease occurred in a group of 110 cockerels 6 to 7 weeks of age very soon after they had been bought. In this group 58 birds died or were killed in extremis to obtain fresh material for post mortem examination. The description of the disease is based on the detailed examination of 38 birds; a further 8 had been submitted for an independent post mortem study to determine the presence of any significant intestinal parasites. Twenty three of the remaining members of the group showed symptoms but recovered; other birds may have been affected with a mild form of the disease which was difficult to detect. The disease appeared in an acute and chronic form, and where necessary this distinction will be made throughout the description of the condition.

Clinical Signs of the Disease

The only clinical signs presented were general signs of malaise as seen in the fowl. In the acute form an apparently healthy bird tended to lose its appetite for about three days and abstained from

food during the last twenty-four hours, during which time the faeces became dry, dark and occasionally blood stained. In the last stages the bird could not stand, the wings and head drooped and the eyes were shut. Death followed within hours.

The chronic form had an insidious onset and the only sign was gradual loss of weight for one to four weeks, sometimes longer, until the bird had lost most of its musculature. The appetite remained moderate until the terminal stages, but there was an increased consumption of water, and the faeces became pale and fluid. No change of diet or addition of vitamin or mineral supplement affected the course of the disease. The majority of the debilitated birds died without any other symptoms, but occasionally birds that were not severely emaciated made a spontaneous recovery.

Pathological Findings

Macroscopic Changes

A detailed post-mortem and histological examination was made of 38 of the affected birds. Some had died, but others were killed in the terminal stages of the disease.

Acute form. There was no significant loss of body weight in birds dying of the acute form, and the liver and spleen were occasionally slightly heavier than those of control birds of comparable breed and body weight.

In the most severely affected birds there was a diffuse hyperaemia of the subcutaneous tissue, and in the foot the second and proximal portion of the third digits were slightly enlarged and intensely congested (Fig. 1).

The crop and small intestine were usually free of food material and no significant changes were observed in the oesophagus, crop, gizzard and proventriculus, or in the first limb of the duodenum, but occasionally the mucosal surface of the second limb of the duodenum was slightly congested. In every case the small intestine had an area of haemorrhagic necrosis of variable length and in the most severely affected portions this was accompanied by extensive dilatation. The lesion sometimes involved a continuous length of intestine from the more posterior part of the jejunum to the middle of the terminal part of the ileum as in Fig. 2, but in other cases involved several areas of the small intestine, 0·5 cm. to 3·5 cm. in length, particularly in the ileum, each portion being sharply demarcated from the adjoining normal bowel. On incising the affected areas, the bowel wall appeared thicker than normal and congested. The lumen sometimes contained fluid contents of a bile-stained haemorrhagic nature, or a thick hard core of granular debris. In some lesions this core was lying free in the lumen, but in others was still firmly adherent to the mucosa. Stained smears of this core revealed no food material, but a mass of degenerate cells, some erythrocytes, and large numbers of Gram positive rods. The caeca occasionally contained blood-stained material, and though the mucosa was congested, there did

not appear to be any necrosis. The mesentery contiguous with the affected portions of intestine was thickened and oedematous and, though the mesenteric vessels tended to be congested, they were sometimes devoid of blood cells for 1 to 2 cm. from the serosa.

The liver was intensely congested and the gall bladder dilated. When the surface of the liver was examined obliquely to a source of light it was occasionally possible to find a variable number of paler areas with sharply defined edges. They measured 3 mm. to 2 cm., and could be found in any part of the organ.

The spleen tended to be congested, but this was difficult to assess in the fresh tissue. No significant changes were observed in any other organ of the body.

Chronic form. Birds dying of the chronic form were in an emaciated condition. The musculature was pale and oedematous, and there was oedema of the subcutaneous tissue and within the peritoneal cavity. The weights of the carcases, livers and spleens, compared with the weights of control birds of similar breed and age are presented in Table 1, together with the volume of bile obtained from the gall bladder.

TABLE 1
BIRDS DYING OF CHRONIC FORM OF DISEASE

	Normal (total 11)		*Chronically affected (total 27)*	
	Average	*Variation*	*Average*	*Variation*
Body weight	1700 g.	1550–1780 g.	960 g.	720–1150 g.
Weight of liver without gall bladder	45 g.	41·5–48·0 g.	23·3 g.	19·8–29·1 g.
Weight of spleen	2·6 g.	2·1–2·9 g.	0·28 g.	0·2–1·8 g.
Volume of bile in gall bladder	2·3 ml.	1·2–2·7 ml.	4·4 ml.	2·9–5·4 ml.

A comparison of the weights of the body, liver and spleen of normal and diseased birds from the fresh carcases of commercial Light Sussex cockerels about 11 weeks of age. Birds dying of the acute condition had body and organ weights similar to those of the control birds.

At the time of death the crop and small intestine were usually free of food material, but the crop and proventriculus often contained fluid lightly stained with bile. The proventriculus tended to be flabby and pale, but the small intestine was always flaccid. In the jejunum and ileum, but never in the large intestine, there were diffuse segments 2 to 5 cm. in length which had a dull grey discolouration, and though dilated, the bowel wall was thickened and stiff when compared with the remaining more normal portions. The lumen of these segments contained small amounts of bile-stained material, and the exposed mucosa was pale and of a granular texture. If such a segment with the mucosa exposed was placed beneath the surface of a bath of physiological saline, and compared with a normal segment from the same bird, the villi of the former

appeared relatively stiff and unyielding when the saline was agitated, whereas the latter had large numbers of flexible villi that moved easily in the stream of saline.

The liver was always small and congested and on close inspection irregularly mottled with paler areas of very variable size which were sometimes difficult to detect. The gall bladder was always grossly distended with viscous bile, and it appeared possible for the bile to penetrate the walls of the bladder, for even in freshly killed carcases the liver, the surface of the anterior part of the duodenum and other contiguous tissues were bile stained (Fig. 3).

The spleen was reduced to a small shrivelled blue-grey remnant, sometimes measuring only 0·4 cm. along its long axis. The heart musculature was pale and without tone, with occasional foci on the epicardium of the ventricles, dull white in colour and about 0·4 cm in size. The pericardium usually contained from 0·75 ml. to 2·5 ml. of a clear, non-viscous, acellular fluid.

The kidneys were often slightly congested but otherwise appeared normal, and the testicles of cockerels were reduced to small atrophic remnants.

Microscopic Changes

Intestine

Areas of degeneration were to be found in the jejunum and ileum. The blind caeca were occasionally slightly affected, but significant changes could not be found in the crop, gizzard, proventriculus or in the first part of the duodenum.

Acute form: early stage. Small, well demarcated areas of degeneration were to be found in the jejunum and ileum, usually separated from each other by normal portions of intestine, even when they coexisted with areas of severe necrosis. The villi were partially or completely denuded of epithelium and large sheets of disintegrating cells were deposited into the lumen (Fig. 4). Those epithelial cells that remained had cytoplasm that could be deeply and homogeneously stained by eosin, and the striated border could no longer be identified. The goblet cells, though exhibiting degenerative changes in the nuclei, retained their mucin content, and when these cells were shed into the lumen each appeared as a ghost cell surrounding an intact globule of mucin. The capillaries of the lamina propria were congested, though several of the larger vessels in this region and the submucosa were occluded by thrombi and were undergoing hyaline degeneration. There was diffuse oedema throughout the affected area and a slight infiltration by fibroblasts, though at the periphery of the lesion fibroblasts were more numerous and there were a few scattered heterophil polymorphonuclear leucocytes. In the centre of these lesions were found droplets or irregular deposits of dense basophilic material, resembling splashes of ink. These deposits stained deeply by the Feulgen technique and this affinity could be removed by prior treatment of the preparation with deoxyribonuclease.

Many of the nuclei of cells within the lamina propria were vacuolated or appeared to be flowing out of the cells, and small droplets formed by the fusion of material from the nuclei of 2 or 3 cells could readily be observed.

Acute form: late stage. The lumen was filled with a core of granular debris comprised solely of a fused mass of material, but on the periphery it was possible to find groups of shed degenerate epithelial cells and erythrocytes (Fig. 5). This core appeared to be formed by the fusion of the degenerate villi undergoing a coagulative type of necrosis involving most of the mucosa, resulting in the formation of an amorphous mass with fibrin deposits and some erythrocytes; this was progressively sloughed off into the lumen. The submucosa was also necrotic, and the tunica muscularis, infiltrated by oedema, contained few nuclei and areas of hyaline degeneration.

In the debris of the sloughed tissue were large numbers of thick Gram positive rods about 4 to 7μ in size lying singly, in irregular groups or short chains of about 5 organisms (Fig. 6). They tended to taper towards each end except for those few bearing spores, and no capsule could be observed, but it was seldom possible to find organisms that were not obscured to some extent by the degenerating tissue. Apart from a few fibroblasts at the periphery, no cellular infiltration was observed and no leucocytes in any part of the lesion.

Chronic form. This was a gradually progressive form in which large areas of the small intestine were affected, and the lesions merged with areas of normal tissue, in contrast to the sharp demarcation of the acute form.

In the initial stages of degeneration all blood vessels were congested and there was a diffuse oedema. Some of the capillaries in the stroma of the villi contained fibrin clots, and any nodules of lymphoid tissue in the lamina propria contained enlarged vacuolated cells with pale nuclei. The epithelial cells of the villi were enlarged, with pale vacuolated cytoplasm, and an indistinct homogeneous deposit in the place of the striated border. The mucus of the goblet cells remained intact even when the cells had completely degenerated, and the argentaffin cells in both the affected and surrounding relatively normal areas had lost their affinity for silver.

At a more advanced stage the process of degeneration progressed down the villi, in some cases in so regular a manner that many villi were affected to an equal extent, and a sharp line separating the necrotic and relatively normal tissue parallel to the muscularis mucosa extended for a centimetre or more. Usually the stroma of the villi remained as an anucleate necrotic fragment as in Fig. 7, but occasionally even the stroma was sloughed off. There was less congestion than in the initial stages and no leucocyte infiltration. The majority of the blood vessels had fibrin with an amorphous, basophilic, Feulgen-positive deposit which in some preparations appeared to be derived from the nuclear material of the endothelium (Fig. 8).

The final stage of degeneration affected all the layers of the intestine from mucosa to serosa, leaving a necrotic anucleate tissue that to a remarkable extent preserved its original form as demonstrated in Fig. 9. The adjoining mesentery was oedematous, with thrombi and basophilic, Feulgen positive droplets in the lumen of the vessels.

The caecum was only mildly affected in these chronic cases. The lymphoid tissue of the tonsil of the caecum had undergone extensive degeneration and only pale remnants of the follicles remained, but the folds and short villi of the mucosa were normal or displayed traces of degeneration and some congestion. The blood-stained material observed in the lumen of the caecum of some cases appeared to be deposits from the small intestine and not the result of degeneration of the caecal wall.

Liver

Necrosis was more extensive in the chronic cases than the acute, as the small focal areas of degeneration observed in the acute form of the disease enlarged and coalesced to involve most of the substance of the liver. In all cases however, the degeneration had the same characteristic appearance.

Lesions could be found in histological preparations of all parts of the organ. They were usually situated on the surface, where the first changes could be observed, but in some material, particularly that obtained from birds dying of the chronic debilitating form, areas of degeneration surrounded all the main blood vessels; occasionally, the whole of the substance in the centre of the organ was affected, leaving fairly intact tissue beneath the capsule. The typical necrotic lesion beneath the capsule was made up of a series of distinct linear zones parallel to the capsule (Figs, 10 and 11) and each zone is described in the order of its appearance commencing from the normal parenchyma.

The normal rather diffuse lobular pattern of the chicken liver was abruptly terminated by a very distinct zone lying parallel to the capsule except where a branch of the portal vein entered the zone, for in such areas the necrotic tissue penetrated the normal parenchyma to surround the vessel. This zone was made up of hepatic cells seven to ten cells in depth, or in some lesions as many as eighteen. The nuclei of these hepatic cells were pyknotic and homogeneous, though a few of the condensed nuclei were distorted into a bizarre shape still containing the remnants of a chromatin network. These nuclei still stained well with the Feulgen technique. The cytoplasm stained deeply and homogeneously with eosin, and in nearly all of them it was no longer possible to demonstrate mitochondria or glycogen. The capillaries were congested but there were never any leucocytes present, and those bile canaliculi that could still be recognised contained pale homogeneous eosinophilic deposits.

The next zone was of variable depth and the semblance of the

original lobular pattern was again apparent. Most of the hepatic cells were about normal in size or slightly enlarged, but had undergone a number of changes. Some of the nuclei were still pyknotic, but many, especially those nearer the capsule, were enlarged, pale and vacuolated. The nuclear membrane often had part of the chromatin network precipitated onto the inner surface, leaving an indeterminate central pale vacuole. The cytoplasm stained very weakly with eosin and contained numbers of small vacuoles which for the most part had no affinity for eosin but stained weakly with the tetrazo reaction for protein. Some of these cells were so distended with their vacuolated contents as to obliterate the sinusoids.

Arterioles and veins in this area had undergone extensive degenerative changes. Erythrocytes, but no leucocytes, persisted in the lumen, and were enmeshed in amorphous basophilic strands of material derived from the dissolution of the endothelial cells of the tunica intima (Fig. 12). This material stained well with Feulgen and the affinity for this stain could be removed by prior digestion of the preparation with deoxyribonuclease. In the severely affected vessels it was not possible to recognise any of the elements of the media which had undergone hyaline degeneration, but in a few vessels sufficient remained to determine smooth muscle elements and, by Weigert's method, elastic fibrils.

The next zone was also of variable depth. The cytoplasm of the hepatic cells became very granular, with fewer but larger vacuoles. The main change appeared in the nuclei which assumed a variety of forms and lost all their normal characteristics. Many persisted as basophilic droplets appearing to flow into different shapes as seen in Figs. 13 and 14. In all fields some of the nuclei had fused, leaving amorphous droplets of basophilic material, often in an extracellular position. These droplets stained well with Feulgen and this affinity could be removed by prior digestion with deoxyribonuclease but not ribonuclease. They did not stain with methyl green. The lumen of all the blood vessels in this zone was also packed with these wefts of deoxyribonucleic acid material, and contained no blood cell elements at all.

This zone of nuclear lysis either merged imperceptibly with the final zone, or there existed a definite demarcation between the two. The last zone extended to the capsule. All the hepatic cells were reduced in size, necrotic and anucleate. The cytoplasm was finely granular with a thick cell wall and usually a large vacuole where the nucleus had been. Large lacunae were left between the distorted cords of cells in which it was not possible to demonstrate any stainable material. These cells stained yellow with McFarlane's modification of Mallory's trichrome technique instead of the normal red colouration of the cytoplasm.

The remainder of the liver was always congested, with a few or no leucocytes, and whereas small nodules of lymphoid tissue may usually be found in the liver of normal chickens, they were rarely

found in preparations obtained from birds dying of this condition. In some preparations there were thrombi in the larger branches of the portal veins, some attached to the endothelium and others free within the lumen (Fig. 15). They consisted of dense fibrin clots containing a few degenerate erythrocytes in the centre, but more on the periphery where there were also a few macrophages densely laden with pigment staining deeply with Perl's Prussian blue. Where the thrombi were found attached to the vessel wall a few fibroblasts were also found, but there was rarely any attempt at organisation. In three severely affected cases, similar mural thrombi were found in the portal vein at the site of its entry into the liver.

In histological preparations from the livers of chronically affected birds, circular areas of necrosis were found surrounding branches of the portal vein. The zones were the same as those described on the surface, but the anucleate necrotic remnants of the hepatic cells comprised the zone adjoining the degenerate remnants of the tunica adventitia, and extending radially through the other zones described, to the normal parenchyma as in Fig. 15.

Gall Bladder

Gall bladders which had been found to be greatly distended on post mortem examination, and which were obtained from birds with extensive liver degeneration, were found on histological examination to be reduced to necrotic acellular sacs. There were no nuclei present. The mucosa had disappeared and the lamina propria and muscular layer were reduced to a fused hyaline fragment in which it was just possible to determine some of the cell outlines; no trace of elastic tissue remained.

Spleen

Histopathological changes in the spleen varied with the severity of the condition. When the animal died a short time after the appearance of clinical symptoms without much bodily wasting, the blood vessels were engorged with erythrocytes. There were fewer cells in the lymphoid follicles, which had pale lymphopoietic centres, and some of the lymphocytes had vacuolated nuclei. A few of these degenerate lymphocytes and erythrocytes had been phagocytosed by histiocytes.

In those animals which were severely emaciated at the time of death and in which the spleen was reduced to a shrivelled remnant, very little of the lymphoid tissue remained. Some of the lymphoid nodules could be recognized, containing small vessels occluded by thrombus formation, but there were many other similar necrotic nodules in which no such vessel could be found and it was not possible to determine their origin. The remnants of the lymphoid nodules were small, with scanty well dispersed cells of very variable and bizarre form. Some had pyknotic nuclei whereas others had vacuolated nuclei with irregular condensations of chromatin

material, and the cytoplasm varied in size, shape and affinity for eosin, though a few developed a weak, diffuse basophilia (Fig. 16). Scattered throughout were irregular droplets of extracellular Feulgen-positive nuclear material. There were always erythrocytes in the sinusoids and the few patent blood vessels were congested, but most of the vessels were occluded by thrombi and had undergone hyaline degeneration, as had the splenic capsule.

In six birds there was a focal, well defined area of gross necrosis involving the capsule and a segment of the tissue beneath it. This lesion corresponded to those found on the surface of the liver, and the outermost part was the more affected.

Heart

The heart was seldom severely affected. Small areas of either ventricle, just under the epicardium, were occasionally degenerate with swelling of the muscle fibres and vacuolation of the nuclei. Even when large amounts of serous fluid were present in the pericardial sac neither the heart nor the pericardium showed extensive changes.

Kidney

The only changes observed were in the kidneys of chronically affected birds, and appeared to be similar to the atrophic changes taking place in many debilitating conditions of the fowl. Occasionally there were small diffuse areas of necrosis in the cortex involving several glomeruli, with vacuolation of the cytoplasm or cellular fragmentation, and the nuclei were invariably pyknotic.

Femur Marrow

Histological preparations of the bone marrow of birds dying of the acute form of the disease were not made, but the marrow of chronically affected birds was observed to be very different from that of normal birds. There were no fat cells present, except that occasionally a few scattered cells remained near the extremities. All the myeloid tissue had disappeared and not one polymorphonuclear leucocyte could be found in any preparation. The marrow consisted of reticular fibres interspersed with small dense nodules of erythrocytes and an occasional giant cell with several centrally situated nuclei, which could not be identified.

Foot

In birds in which congestion and necrosis of the terminal digits were observed, as in Fig. 1, histological examination revealed an intense hyperaemia of the vessels associated with the second and third digits. Some of the smaller vessels were occluded by thrombi, and there was localised oedema and diffuse haemorrhage with the result that these areas stained metachromatically with toluidine blue. Few or no leucocytes could be found, but there were a number of fibroblasts.

TABLE 2
HAEMATOLOGICAL FINDINGS

Condition of bird	*Number of birds*	*Total packed cell volume %*		*Leucocyte-thrombocyte fraction %*	
		Average	*Variation*	*Average*	*Variation*
Normal	5	28·7	26·9 – 30·3	2·3	2·1 – 3.0
Acute form	3	26·4	22·3 – 28·2	1·1	0·4 – 1·7
Chronic form	16	19·8	16·9 – 23·7	0·4	0·1 – 0·7

Haematocrit readings obtained from blood samples centrifuged at 3000 r.p.m. for 30 mins. Two tests were carried out on the blood of each bird, and the samples were obtained from birds affected by the acute and chronic forms of the condition, and clinically normal birds from the same group.

Skin

In the few subjects in which congestion of the subcutaneous tissues was observed, microscopic examination revealed congestion of the vessels of the dermis, especially beneath the papillae of the feather follicles.

Haematological Investigations

The total packed cell volume and the white cell-thrombocyte fraction are presented in Table 2. After the haematocrit readings had been recorded, the white cell-thrombocyte fraction was removed by a fine pipette, resuspended in 10 per cent chicken plasma in saline, and smears were prepared for microscopical examination. There were very few leucocytes in this fraction obtained from blood of birds in extremis in the chronic form of the condition, the bulk of the cells being aggregated thrombocytes and some amorphous debris. Birds dying of the acute form also had a great reduction of leucocytes, especially mononuclear cells, when compared with clinically normal birds of the same group.

Bacteriological Investigations

No bacteria of significance were isolated from the intestine or the internal organs of affected birds by culture under aerobic conditions. Despite the gross macroscopic changes observed in several birds the organs were usually sterile, and culture media, stained smears of the tissues and histological preparations failed to provide evidence of P.P.L.O., or vibrios.

A filterable agent capable of causing cytopathic changes in monolayer cell cultures was recovered from the faeces of two of the birds, but appeared unrelated to the disease since it caused no specific lesions or deaths in chick embryos and no disease in adult birds when administered orally or injected intraperitoneally.

In 27 out of 34 birds in which anaerobic incubation of the culture media was carried out, an organism which proved to be *Cl. welchii* was isolated from the intestine. It was found in the small

intestine in every case examined where there was a deposit of necrotic cells in the lumen. In some birds, particularly those affected by the acute form of the condition, the organism could be isolated from the faecal material present in the large intestine, but was rarely isolated from the contents of the blind caeca. The organism was never isolated from any other organ, even if as much as a gramme of the tissue was triturated and the whole amount cultured in broth or meat media. A description of the characteristics of this strain of *Cl. welchii* is presented in another paper (Parish, 1961a).

Saline extracts of the contents of the small intestine of birds dying of the acute form, filtered through a gradocol membrane with an average pore diameter of 0·4μ, were capable of killing young chickens when 1·0 ml. volumes were injected intravenously. These extracts would kill mice within 12 min. when 0·2 ml. was injected intravenously. Incubation of the extracts of the intestinal contents with *Cl. welchii* type B and type C antitoxic sera neutralized the toxicity of the extracts for mice, but the extracts remained lethal for mice after incubation with type A and type D antitoxic sera.

In only 3 of 7 chronically affected birds was it possible to extract a mouse lethal toxin from the contents of the small intestine and in each case the lesions in the small intestine were severe. It appeared that the toxin could only be recovered from the contents of the small intestine if severe changes were present, even if the strain of *Cl. welchii* could be recovered from the intestine of these birds. In no instance (7 attempts) was it possible to extract mouse lethal toxin from the liver or spleen.

DISCUSSION

The disease occurred in a large group of cockerels which had been selected as being well matched in weight and were purchased when they were 6 to 7 weeks of age. They were fed a proprietary compound meal mixture, and additional vitamins A and D in powder form were supplied every week. The first bird died with the acute form of the disease on the fifth day, and others died at intervals over a period of 3 months, though after the first month death from the acute form was unusual. No change of diet nor supplements of vitamins and mineral salts affected the course of the disease, and other birds of comparable age obtained from a different source, yet maintained in the same environment and on the same food, remained unaffected. Carcases submitted for an independent post mortem examination were reported to be free of coccidiosis.

A strain of *Cl. welchii* was eventually isolated from 27 out of 34 birds in which material from the affected areas of the small intestine was cultured anaerobically. In those subjects in which there were severe necrotic lesions of the small intestine large numbers of these organisms could be isolated in apparently pure culture. Whatever the chemical nature of the changes taking place in the necrotic tissue of the intestine, it appeared that they did not induce conditions

suitable for secondary infection. The in vitro characteristics of this strain have been examined (Parish 1961a), and they appear be to similar to those of *Cl. welchii* type F. It was possible to reproduce the condition in a few birds with organisms that had been sub-cultured several times on laboratory media (Parish 1961b). Other evidence in support of the contention that *Cl. welchii* was the causal agent was the isolation of a toxin or toxic complex from the contents of the small intestine which was lethal on intravenous injection into mice and could be neutralized by *Cl. welchii* type B and type C antitoxin. Nevertheless there is no proof that this organism of itself is the cause of the disease for no data are available of the incidence of *Cl. welchii* type F organisms in the intestinal contents of normal birds; in addition there is no evidence as to whether *Cl. welchii* toxin is present in the intestine of normal birds. The disease was induced experimentally with this organism only with difficulty, and unknown predisposing factors appeared to be essential.

The areas of necrosis observed at post mortem examination appeared to be more severe in the small intestine than in the liver and spleen, especially in the acute form of the disease, where it was not easy to recognise changes in the liver other than congestion in birds having gross necrotic areas in the intestine. The histological changes in the liver were more severe than were anticipated from the macroscopic appearance, but in both the acute and chronic forms, areas of necrosis in the substance of the liver were associated with the course of the branches of the portal veins, and thrombosis of the veins was a common feature. These changes may be interpreted as resulting from an enterotoxaemia, in which the toxic agents were absorbed into the portal system and caused necrosis in the liver. There is no doubt that some agent capable of causing focal necrosis was absorbed into the blood stream, for focal areas of mild degeneration were occasionally found beneath the epicardium of either ventricle, and in a few birds dying of the acute form there was congestion of the subcutaneous tissue and the terminal digits of the foot. Moreover there appeared to be a selective destruction of the lymphoid and myeloid elements of the spleen and bone marrow in the chronic form, which would appear to have been caused by a blood-borne toxin.

There remain however two sites in which necrosis could be found which cannot be explained by haematogenous spread of a toxin. These were the areas on the surface of the liver and the surface of the spleen. Where a lesion was observed surrounding a vein in the substance of the liver, the necrotic area lay next to the vessel wall, and the lesions radiated outwards with differing degrees of degeneration into the normal parenchyma. In those lesions observed on the surface of the liver, the necrotic area comprised the capsule and the area beneath it, and the earlier stages of degeneration lay in the deeper parenchyma. This is suggestive evidence that the toxic factor was also present in the peritoneal cavity and acted

directly on the liver tissue. This explanation could also account for the segments of necrosis on the surface of the spleen which may have been contiguous with an intestinal lesion.

There were three significant findings observed on histological examination of the lesions. The first was the sharp, very definite demarcation between the normal and the degenerating tissues in the lesions of the small intestine and liver. Though the edges of the lesions in the intestine in the chronic form tended to be diffuse, the process of degeneration progressed down the villi, affecting many of them to an equal extent. In the acute form, an area of necrotic tissue in the process of being sloughed off the mucosa was clearly separated from the surrounding area of relatively normal tissue. This sharp separation between normal and degenerating tissue was clearly seen in the liver, and in the lesions beneath the capsule it was so straight that the line could have been set with a ruler.

The second significant finding was the dissolution of the nuclei in many of the lesions. Nuclear material appeared to flow out of the individual cells and become fused in irregular deposits, which on the periphery of the areas faded and disappeared. Apart from the process being observed in the histological preparations, this material was undoubtedly substance from the nuclei as it consistently stained by the Feulgen technique for deoxyribonucleic acid, and was equally consistently digested away by the enzyme deoxyribonuclease, but not by ribonuclease. The early loss of the affinity of these affected nuclei for methyl green was probably due to the greater capacity of Feulgen to react with lower polymers of deoxyribonucleic acid.

The third finding, probably related to the second, was the failure to find any infiltration of leucocytes into the degenerating and necrotic lesions; an exception to the normal processes of inflammation. This was to some extent accounted for by the low number of leucocytes present in the circulating blood and, in the chronic form, by the degeneration of the leucocyte-forming areas of the spleen and bone marrow. Unfortunately it was not possible to obtain accurate total and differential counts of the circulating leucocytes by methods described in the literature and only the haematocrit method produced consistent results in these experiments. Nevertheless it appears that some toxic agent selectively destroyed the lymphoid and myeloid elements of the body and inhibited infiltration of the leucocytes into the lesions, though the nucleated erythrocytes appeared resistent to the toxin.

All these necrotic changes should reflect the state of the tissues at the time of the death of the birds, because many of those used for detailed histological investigation were killed when in extremis, post mortem examination carried out immediately and samples of the tissues immersed in rapidly acting fixatives.

The evidence so far adduced lends support to the hypothesis that a strain of *Cl. welchii* elaborated toxins in the lumen of the small

intestine, and that these caused severe local necrotic lesions of the mucosa. Only on a few occasions were the conditions suitable in the small intestine for large portions of it to be colonised by the organism. These toxins were capable of causing rapid or protracted death of the affected bird. In the process, focal areas of degeneration appeared in the liver, characterised by necrosis and karyolysis; in the spleen and bone marrow the germinal centres of leucocyte formation were destroyed.

A form of enteritis in man termed 'enteritis necroticans' or 'Darmbrand', was first described in Germany by Beckermann and Laas (1946) who reported seven cases, which however could be divided into two separate groups. The essential histological features were necrosis of areas of the small intestine, and occasionally the large intestine, with normal tissue remaining between the areas of degeneration from which the necrotic areas were sharply demarcated by a straight line. The necrotic tissue was sloughed off to expose the haemorrhagic and oedematous areas of the submucosa and musculature. The manner in which much of the necrotic tissue preserved its form was described as noteworthy. Thrombi were observed in the vessels of the mucosa, and leucocytic infiltration in the demarcated areas.

Jeckeln (1947) provided a more critical study of the histopathology from which the following relevant details have been selected. The lesions were observed in the small intestine where necrosis was the chief feature leaving a shadow of the tissue without nuclei, and the cellular elements of the oedematous exudate were conspicuously scanty. In the early stages there was necrosis of many villi without any marked cellular reaction in the periphery of the dead region, and where the necrosis penetrated deeply the vessels of the small intestine were thrombosed. Later the necrotic mucosa was sloughed off and the necrosis penetrated deeper into the submucosa and musculature. Siegmund (1948) described much the same changes, but laid more stress on changes in the blood vessels of the intestine which were observed to have fresh fibrinoid deposits in the lumen. No attempt was made to determine the nature of this material.

The lesions in the small intestine of the fowl differ from the descriptions cited above in that there was no leucocytic infiltration except in the very early stages, whereas Beckermann and Laas (1946), Jeckeln (1947) and Siegmund (1948) describe it as being present, usually scanty, but sometimes profuse in the zone of demarcation at the periphery. It is possible that the total white cell count could reflect this difference, for in the human cases there was usually a leucocytosis of 16,000 or more cells per c.mm. (Beckermann and Laas, 1946; Ruppert, 1947). In the fowl there was an early and persistent leucopenia. No significant lesions were described in the liver or spleen of the human cases and no mention made of the phenomenon of karyolysis in these organs.

The necrotic lesions described in the small intestine of man are similar to those found in the fowl, and as the nature of the condition is essentially necrosis, the term necrotic enteritis is considered to be justified. Furthermore the characteristics of the type of *Cl. welchii* isolated from these birds (Parish, 1961a) appear to be the same as those described as typical for *Cl. welchii* type F., which was isolated from the human disease (Zeissler, 1949; Zeissler and Rassfeld-Sternberg, 1949; Willich, 1949).

To the knowledge of the author, gross and extensive necrosis of the liver without leucocytic infiltration or fibrosis has not been previously described in the fowl. The lesions described in infectious avian hepatitis as resulting from infection by vibrios are infiltrated by both leucocytes and fibroblasts (Sevoian, Winterfield and Goldman, 1958; Hofstad, McGehee and Bennett. 1958; Casoroso and Jungherr, 1959), and in the condition first described as 'alimentary toxaemia of chickens' resulting from the presence of a high percentage of fat in the diet, the liver necrosis was accompanied by infiltration of heterophils, petechiae and prominent fibrosis (Sanger, Scott, Hamdy, Gall and Pounden, 1958). One other condition reported by Marthedal (1953) was characterised by necrotic foci and congestion of the liver of young chickens and focal areas of congestion in the caeca and rectum. On microscopic examination the liver was found to have lobules, or larger foci, with regressive and degenerative changes scattered in normal parenchyma. No infectious agent could be isolated and it was regarded as a sequel to a nutritional disturbance.

CONCLUSIONS

An enterotoxaemia in fowls apparently caused by a strain of *Clostridium welchii* is described. The condition occurred in an acute and a chronic form. In the acute form there were areas of haemorrhagic necrosis of the small intestine, and congestion and degeneration of the liver. Birds affected by the chronic form were emaciated, with degeneration of the liver, spleen and bone marrow, and there were diffuse areas of degeneration without haemorrhage in the small intestine. There was a drop in the total packed blood cell volume of these affected birds, and the leucocyte-thrombocyte fraction was much lower than normal.

On histological examination the lesions consisted of areas of necrosis, usually sharply demarcated from the normal tissue, and in the liver there were a series of well-defined zones varying in degrees of degeneration. There were few or no leucocytes in any of the lesions, and in the chronic form there was destruction of the lymphoid tissue of the spleen and myeloid tissue of the bone marrow. Another significant feature was the karyolysis of the nuclei of cells of the liver and, in a few cases, of the intestine resulting in the deposition of deoxyribonucleic acid within the tissues.

A toxin was extracted from the contents of the small intestine

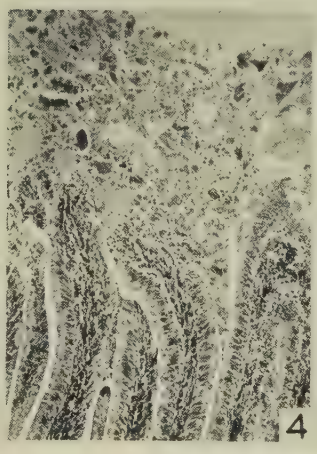


Fig. 1. Foot of cockerel with haemorrhagic necrosis of the terminal digits.
Fig. 2. Intestine of cockerel with the acute form of the disease. There is haemorrhagic necrosis of the ileum and dilatation of the terminal part.
Fig. 3. Fresh carcase of cockerel with the chronic form of the disease. The distended gall bladder lies to the right of the liver and the mesentery beneath is bile stained.
Fig. 4. Villi of ileum in the early stage of the acute form of the disease. Haematoxylin and Eosin x 45.

To face page 392

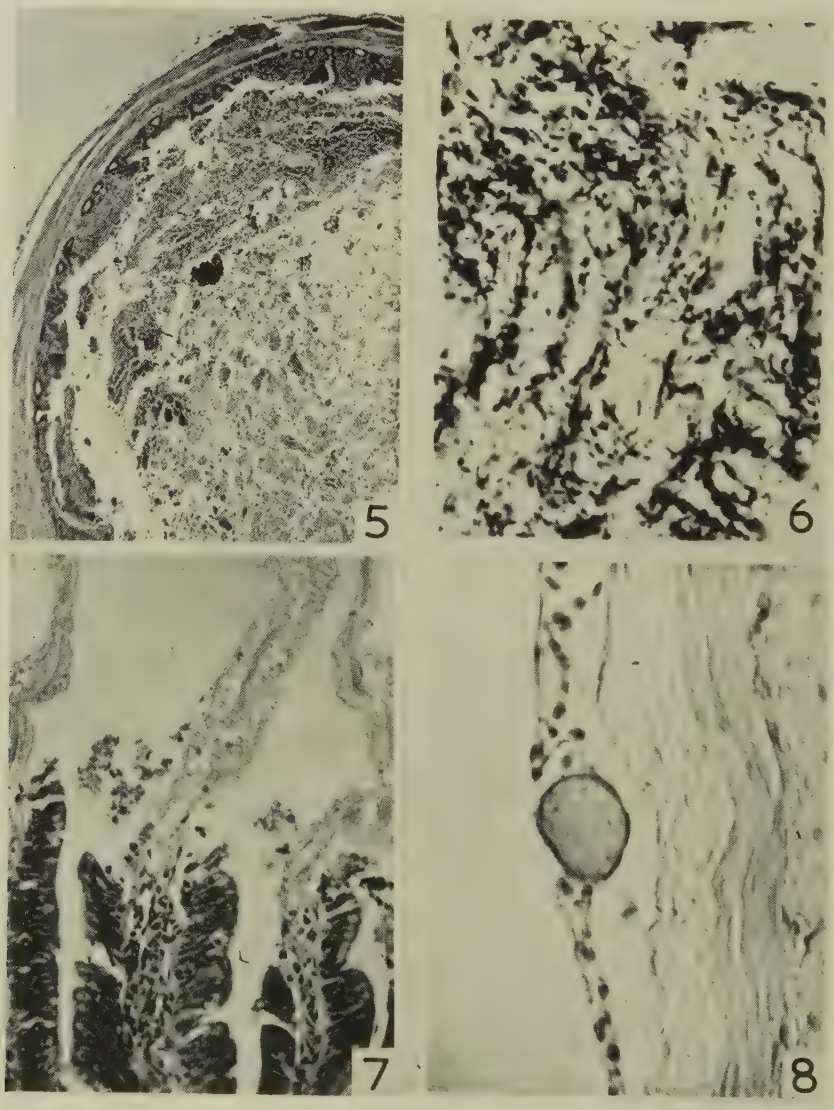
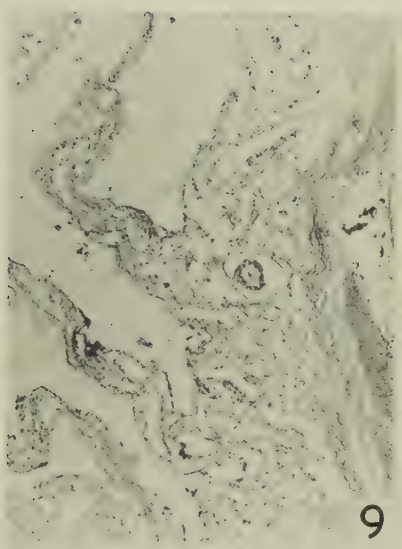


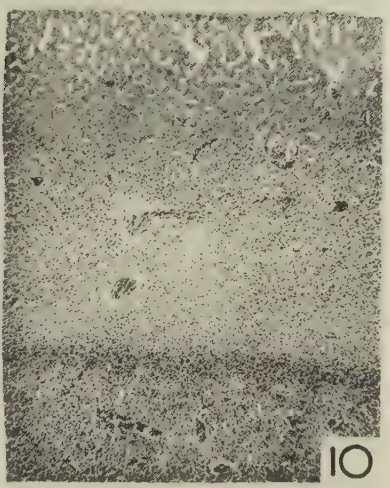
Fig. 5. Gross necrosis of the terminal ileum in the late stage of the acute form of the disease. H. & E. x 33.

Fig. 6. Bacterial organisms associated with necrotic remnants of the villi of the ileum. Gram x c. 400.

Fig. 7. Villi of ileum in early stage of degeneration in the chronic form of the disease. H. & E. x 150.

Fig. 8. Serosa of ileum with a blood vessel filled with an amorphous deposit. Feulgen x 280.





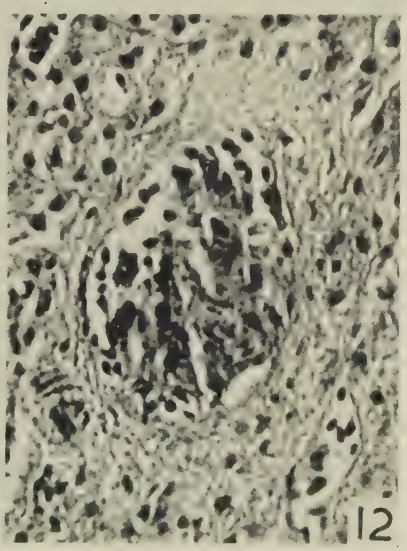
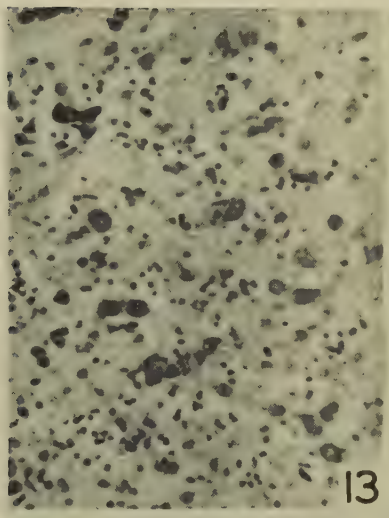


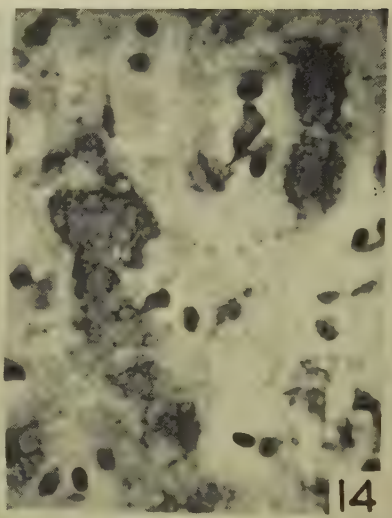
Fig. 9. Final stage of necrosis of the ileum in the chronic form of the disease. Only the condensed cytoplasm of the connective tissue persists. H. & E. x 27.

Fig. 10. Necrosis of surface of liver. Area beneath capsule is the most severely affected of the 4 zones of degeneration. 2 blood vessels in the area of degeneration contain amorphous basophilic material. The normal parenchyma at the bottom of the fig. is sharply limited by the first zone of degeneration. H. & E. x 53.

Fig. 11. Necrosis of surface of liver. The normal parenchyma at the bottom of the fig. is sharply limited by the first zone of degeneration. In this fig. the zones are closer and more distinct than in Fig. 10. The fused basophilic deposits following karyolysis of the nuclei may be seen in the third zone. H. & E. x 110.

Fig. 12. Blood vessel of liver containing erythrocytes enmeshed in diffuse basophilic material apparently derived from the endothelial wall. H. & E. x 280.





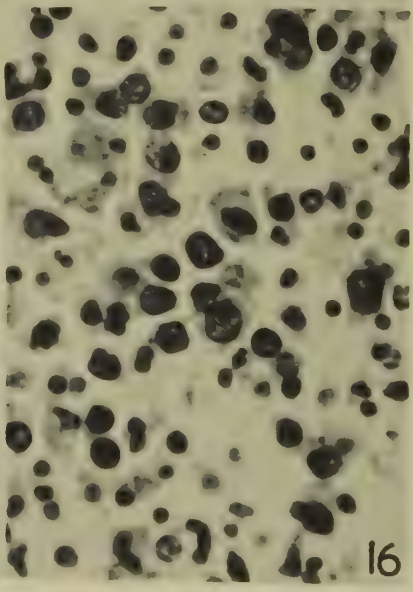


Fig. 13. Area of degeneration in the liver with karyolysis of the nuclei of the hepatic cells. H. & E. x 150.

Fig. 14. Enlargement of an area of fig. 13 to demonstrate the karyolysis of the hepatic nuclei and fusion of the nuclear substance from groups of cells. The nuclei of the erythrocytes are not affected. H. & E. x 510.

Fig. 15. Thrombus in branch of portal vein in the liver. The vessel is surrounded by an area of degeneration resembling that observed in figs 10 and 11. H. & E. x 60.

Fig. 16. Lymphoid nodule of spleen. The small number of surviving cells have an irregular morphology and the nuclei are in various stages of degeneration. H. & E. x 850.

of birds dying of the acute form which was lethal to mice and young chickens on intravenous injection. This toxin could be neutralised by *Cl. welchii* type B or type C antitoxins.

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NECROTIC ENTERITIS IN THE FOWL

II. Examination of the Causal *Clostridium welchii*

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INTRODUCTION

The strain of *Clostridium welchii* isolated from the chickens dying of necrotic enteritis described in the previous paper (Parish, 1961) was examined to determine its identity and to attempt to isolate the toxic principle responsible for the nuclear karyolysis observed in the intestine, liver and spleen.

The organism under investigation is referred to as the test organism throughout this paper, to avoid designating it by a number or letter that may cause confusion with the accepted nomenclature.

MATERIALS AND METHODS

The organism was cultured routinely on sheep blood agar incubated anaerobically. It was thereafter preserved on alkaline egg medium at +4°C and in Robertson's meat medium at room temperature, and though subcultured every month, the original cultures remained viable for four months. Sugar fermentation tests were carried out in the normal manner and the final pH was checked by a Cambridge potentiometer. Antibiotic resistance tests were performed in similar tubes in both glucose and beef broth, each antibiotic being dissolved in sterile distilled water and added to the medium to give the final required concentration an hour before the tube was inoculated. After 3 days incubation at 37°C, 0·1 ml. of each tube was subcultured on to sheep blood agar and to 20 ml. glucose broth and incubated 3 days. Spore formation was irregularly induced in nutrient broth by first incubating anaerobically at 37°C, leaving at room temperature for seven days and finally, after bubbling CO^2 through the media, leaving the cultures in an atmosphere of saturated CO^2 for a further 3 days. To test the effect of boiling on these spores, concentrated suspensions of the spore-bearing organisms were made up in 0.25 ml. phosphate buffered saline and added to 4.75 ml. amounts of beef broth preheated to 100°C in a water bath in cotton wool stoppered tubes. Tubes were removed after 30 min. and hourly at 1 to 4 hours, the pH adjusted to 7·5 followed by incubation at 37°C for 14 days.

Toxins were isolated by culturing the organisms on the medium described by Robertson and Keppie (1941). 100 ml. volumes of media at 37°C were inoculated with 1 ml. of a 15 hour meat broth culture, which had been seeded with one colony from sheep blood agar. The pH was maintained at 7.0 and tested at 3, 5, 10, 15 and 20 hours, and 3 days at which times incubation of samples was terminated, the pH adjusted to 7.4 with NaOH and the cultures centrifuged at 4,000 r.p.m. for 2 hours until clear; toxicity tests were carried out immediately. Early samples were filtered through 0·4μ gradocol membranes, but sterility tests showed

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that this was unnecessary. To activate any ϵ toxin present, samples of the fresh toxin were treated with 0.02 per cent. commercial trypsin for 45 min. at 37°C.

Lecithinase tests were performed on human serum and egg substrates. The test on sterile human serum was carried out as described by Mackie and McCartney (1953) using the author's serum in 0.3 ml. vols. The egg substrate, lecithovitellin, was prepared as described by Macfarlane, Oakley and Anderson (1941) as a homogenized filtered egg yolk extract in saline, and any dilutions of toxin were made in saline with the addition of borate-buffer saline, pH 8, and the test performed by the method of Oakley and Warrack (1953). Haemolysis tests were carried out on 2 per cent. suspensions of horse, ox, sheep, rabbit and chicken cells washed 3 times in Dale's Ringer solution. 0·5 ml. volumes were mixed with an equal volume of toxic filtrate, incubated at 37°C for 1 hour, examined and left at room temperature overnight.

Neutralisation tests were carried out in all these experiments using commercial horse antisera (Parish, 1961).

Gel diffusion tests were carried out as described by Elek (1949). Strips of filter paper about 1 cm. in breadth were dipped in the appropriate antitoxin and sunk just beneath the surface of sheep blood agar and horse serum agar plates. The test organism and the control strain were streaked in parallel lines on the surface, incubated at 37°C anaerobically for 48 hours and then left at room temperature 5 days to allow the precipitin pattern to develop.

Deoxyribonuclease estimations of the sterile tissue filtrate were carried out as described by Warrack, Bidwell and Oakley (1951). Other details are given in the text. All dilutions, however, were made in Dale's Ringer solution containing 0.35 g. $MgCl_2$/litre. Preparations were stained by Leishmann instead of Jenner's method, but it was found that since the Feulgen stain was specific for deoxyribonucleic acid it provided more information of the nature of the changes in the test leucocytes. Leucocytes from man, rabbit, sheep and chicken were employed.

Toxoid was prepared from the toxic filtrates of one strain each of types A, B, C, D, E and the test organism, by the addition of formalin to 50 ml. amounts of fresh toxic filtrate to give a final concentration of 0·4 per cent., and the flask sealed and incubated at 37°C. After 5 days and every subsequent 3 days the toxicity was tested by mouse inoculation, and if necessary the concentration of formalin increased by 0·05 per cent; when proved to be non toxic (about the 11th day) the toxoid was dialysed at +4°C against phosphate buffered saline and centrifuged. Alum precipitation of the toxoid with a 10 per cent. solution of potassium alum added dropwise to a final concentration of 2·5 per cent. of the total was used to prepare the antigens for active immunization of guinea-pigs. The precipitate was washed three times in a large excess of saline, and injected intramuscularly as a 10 per cent. suspension and 0·35 ml. volume in 450 g. guinea-pigs. Three injections were given three weeks apart, and the challenge dose intramuscularly in the thigh on the unvaccinated side 21 days later.

Animal inoculation tests to determine toxicity of the sterile toxins and the products of neutralisation by antitoxin were carried out intravenously on mice weighing between 18 and 22 g. and young chickens weighing 750 to 850 g. Guinea-pigs were injected intradermally with 0·2 ml. vol. of toxin or toxin-antitoxin mixture.

RESULTS

Morphology

The organism displayed the typical characteristics associated with *Cl. welchii*. It would only grow on the surface of solid media under anaerobic conditions, appearing as small slightly convex colonies with an entire edge at 24 hours on serum agar, but after 72 hours about 40 per cent. of the colonies were flat with a rough surface and crenated edge, the remainder being convex and smooth, with an entire edge. Subculture of either type of colony produced both colonial forms in about the same proportion.

Examination of smears of the organism from serum agar plates revealed thick Gram positive rods, about 5 to 7μ in length with slightly rounded ends. They remained as single organisms, or in groups of two or three loosely attached either in chain or palisade form, but after six days incubation in some colonies filaments of 4 to 12 cells were found and the junction between each pair of adjoined cells was clearly demarcated. No capsule could be recognised, and the organism was non-motile whether tested on agar slopes or by direct observation or in Craigie tubes. Spores were obtained in any number only with difficulty in culture and were large, oval and subterminal, or occasionally central in position.

Acid and gas were produced in the following sugars: glucose, lactose, sucrose, maltose and salicin. There was no growth in inulin, mannitol, dulcitol or glycerol. Typical 'stormy' fermentation was observed in iron litmus milk in 24 hours, gelatin was liquified in 48 hours, Robertson's meat medium remained undigested and developed a light pink colouration, while both coagulated serum and egg medium supported growth without being digested.

Sensitivity to Antibiotics

Growth was entirely inhibited in glucose or beef broth cultures with the following final dilutions of the antibiotics:—penicillin at 10, 50 and 100 units/ml., and streptomycin, terramycin, aureomycin and erythromycin at 150 μg/ml. When, however, 0·1 ml. of the beef broth culture containing 10 units/ml. penicillin was added to 20 ml. glucose broth, growth was observed on the third day, indicating that growth in the presence of the antibiotic had been arrested but that viable organisms survived.

Thermostability of Spores

Great difficulty was encountered in obtaining spores of the test organism and *Cl. welchii* types A, B, C, D and E. Sometimes large numbers of spores were formed spontaneously in a tube of medium; on two occasions a culture of type A produced large numbers of spores on coagulated serum kept for 6 weeks at room temperature, but this could not be reproduced as required. Eventually bubbling CO_2 gently through an old culture was found to induce spore formation sufficient for testing, but seldom in very large numbers.

Heating suspensions containing spores at 100°C in beef broth resulted in death of the organisms of types A, B, C, D, and E within 30 minutes: the test organism survived 2 hours but not 3 hours.

Detection of Haemolysis

Weak haemolysis was observed on sheep blood agar plates after 2 days anaerobic incubation, but became more noticeable after the plates had been left overnight at room temperature, when a small zone of clear haemolysis with a faint green periphery appeared round the colonies. There was no haemolysis on horse blood agar plates under these conditions or after 6 days anaerobic incubation.

Fresh, centrifuged, unfiltered toxin preparations obtained after 5 hours to 3 days incubation of the test organism, as described in

TABLE 1

HAEMOLYTIC TESTS

Time of culturing before harvesting toxin	*2% suspension of cells in 0·5 ml. Ringer solution*	*Dilution of fresh toxin in Ringer solution tested in 0·5 ml. volumes*				
		Neat	*1/2*	*1/4*	*1/8*	*1/16*
5 hours	Sheep	*t*	*t*	—	—	—
	Rabbit	(+)	t	—	—	—
10 hours	Sheep	+	(+)	t	t	—
	Rabbit	+	+	t	t	—
15 hours	Sheep	+	(+)	(+)	t	—
	Rabbit	+	(+)	t	t	—
20 hours	Sheep	(+)	t	t	—	—
	Rabbit	+	+	(+)	t	—
3 days	Sheep	t	t	t	—	—
	Rabbit	(+)	(+)	t	t	—

The titre of haemolysis obtained by different preparations of the fresh toxin after incubation at 37°C for 1 hour and at room temperature overnight.
+ = about 50% cells haemolysed; (+) = less than 50% cells haemolysed.
t = trace of haemolysis.

Materials and Methods, were tested for their ability to lyse 2 per cent. suspensions of red cells of four species. The details and results of tests on the sheep and rabbit cells are presented in Table 1. The horse and chicken erythrocytes were not lysed. When 1 ml. amounts of the toxin were incubated at 37°C with 0·5 ml. of a 1/2 dilution of *Cl. welchii* type A antitoxin containing 150 units antitoxin/ml. for 30 minutes, the haemolytic activity for sheep and rabbit cells was neutralised. Haemolysis was not neutralised by the addition of 0·5 ml. of a similar dilution of normal inactivated horse serum.

Lecithinase Tests

The organism displayed lecithinase activity as measured by the modified Nagler reaction on human serum diluted in equal parts

of Fildes' peptic digest broth. Inoculation of the medium with one drop of fluid culture of the test organism and incubated anaerobically for 48 hours produced a yellow curd and turbidity. This did not occur in the tube to which 0·03 ml. of *Cl. welchii* type A antitoxin was added to the substrate prior to inoculation of the organism.

Tests were made on the same substrate with fresh toxic filtrate. This had a low titre of lecithinase activity when 0·3 ml. of the toxin dilution was incubated with 0·5 ml. of the substrate at 37°C for 3 hours, the end point being determined as the lowest dilution producing a yellow curd on the surface irrespective of the turbidity of the serum. The toxic filtrate also produced opalescence and slight flocculation on the lecithovitellin substrate within 3 hours at 37°C.

TABLE 2

LECITHINASE TESTS

Time of culture before harvesting toxin	*Substrate 0·5 ml. egg yolk or human serum*	*1 ml. dilution of fresh toxin in saline solution + 0·5 ml. borate buffer saline pH 8*				
		Neat	*1/2*	*1/4*	*1/8*	*1/16*
5 hours	egg yolk	+	(+)	(+)	—	—
	serum	t	—	—	—	—
10 hours	egg yolk	+	+	+	(+)	t
	serum	+	t	t	—	—
15 hours	egg yolk	+	+	(+)	(+)	t
	serum	+	t	—	—	—
20 hours	egg yolk	+	(+)	t	—	—
	serum	+	t	t	—	—
3 days	egg yolk	+	(+)	(+)	t	—
	serum	(+)	(+)	t	—	—

The final dilution of fresh toxin causing flocculation in the substrate after 3 hours incubation at 37°C.

+ = deposit on surface and turbidity of media. (+) = turbidity of media. t = slight turbidity of media.

Neutralisation tests using *Cl. welchii* type A antitoxin had to be discounted since a similar volume of normal inactive horse serum was also able to inhibit this property of the active toxin. The results of the tests of the toxin on these two substrates are presented in Table 2.

Gel Diffusion Tests

All tests were carried out concurrently on sheep blood agar and horse serum agar plates, and several were repeated two or three times. Precipitin lines were sometimes difficult to observe accurately in the blood agar plates.

When the test organism was streaked in parallel with the *Cl. welchii* type A against anti-A antitoxin, the type A strain produced

one dense band which limited a zone of clear haemolysis on the blood agar plate, and in some plates, two faint lines more removed from the barrier of antibody. The test organism produced a single faint line which linked exactly with the dense band produced by the type A strain and was presumably α toxin. Dense precipitin bands were formed by type B and type C organisms against their homologous antitoxin sera. The type C only produced one precipitin line, but the type B tended to produce two, though the second band was fainter and was masked if a heavy precipitate was formed in the major band. The test organism also produced a strong band concurrent with those produced by the type B and C strains. This was more significant when concurrence of this precipitin pattern was obtained on culturing all three strains in parallel against the anti-B antitoxin or anti C antitoxin, and was therefore an indication of the presence of β toxin. No precipitin reaction was obtained by the test organism cultured across a zone of anti D and anti E antitoxic sera.

The test organism therefore had a weak α and a strong β component.

Deoxyribonuclease Tests

Thin films of leucocytes obtained from man, rabbit, sheep and chicken dried in air and fixed in methyl alcohol were found to be equally effective as indicators of deoxyribonuclease activity of the fresh toxin.

Half of each slide was left upright in the dilution of toxin at 37°C for 18 hours and the upper half of the slide left exposed to the air acted in some measure as a control. It was found that staining the slide after exposure to the toxin by Leishmann's technique provided better definition of the nucleus and cytoplasm, but the Feulgen technique was a more useful indicator of the presence of residual deoxyribonucleic acid. Leucocytes would still retain demonstrable nuclei by the Leishmann stain when they appeared as pale blue ghosts by the Feulgen method. By using an end point of gross degeneration of 50 per cent. of the total cells stained by Leishmann's technique a fairly close correlation was found with the dilution of effective toxin that almost completely removed staining affinity for Feulgen. It was noticeable that the polymorphonuclear leucocytes were more resistant than the lymphocytes, and that the nuclei of the chicken erythrocytes were depolymerised.

The toxin obtained from the test organism contained a high activity, and the undiluted toxin was capable of completely destroying the exposed film of leucocytes within minutes. The dilution of the toxin in magnesium-fortified Dale's Ringer solution which produced gross degeneration of 50 per cent. of the test cells varied with the time of incubation of the culture before the toxin was harvested. The toxin obtained after 5 hours incubation had an end point of 1/1024; after 10 hours 1/1024 and in some tests 1/2048; after 15 hours and 20 hours 1/512 and after 3 days 1/16.

1 ml. samples of toxin incubated with 2 ml. of undiluted antitoxin of *Cl. welchii* types A, B, C, D for 37°C for 1 hour lost none of their activity.

Inoculation of Animals

The toxin injected in 0·2 ml. amounts intravenously into mice killed them within minutes. As mice injected with the diluted toxin often lingered for some hours, the M.L.D. was selected as that dilution which would kill all members of a group of 5 mice within 24 hours.

The clinical symptoms and post mortem findings of these mice were typical of death caused by *Cl. welchii* toxins and need no further elaboration, except that mice lingering 12 hours or more had a blood-stained exudate in the peritoneal cavity and the urine also appeared blood-stained. When quantities of these fluids were centrifuged, however, there was little or no haemolysis, the erythrocytes being deposited intact. Neutralisation tests carried out on 1 ml. volumes of the undiluted toxin with an equal volume of antisera undiluted, or diluted at 1/10, and incubated 37°C for 30 mins. resulted in inhibition of mouse pathogenicity by anti-B and anti-C antitoxins, but not by anti-A nor anti-D.

TABLE 3

CONCENTRATIONS OF TOXIN

Time of culture before harvesting toxin	*M.L.D. for mice in ml.*	*M.L.D. for chickens in ml.*	*M.N.D. for guinea-pigs in ml.*
5 hours	0·001	0·25	0·0005
10 hours	0·001	0·25	0·0001
15 hours	0·0025	0·25	0·0001
20 hours	0·0025	0·5	0·0001
3 days	0·025	0·75	0·005

The results were recorded 24 hours after inoculation.

Young chickens were also susceptible, though greater concentrations of the toxin were required to produce death in groups of 5 in 24 hours after intravenous injection.

Intradermal injection of 0·2 ml. toxin in guinea-pigs resulted in extensive necrosis within 24 hours and this test proved to be more sensitive than the mouse pathogenicity tests. The toxic effect on both the guinea-pigs and chickens could be neutralised by type B and type C antitoxins. Prior incubation of the toxin with 0·02 per cent. commercial trypsin slightly reduced the toxicity of the preparation for mice. The results of these experiments are presented in Table 3.

Intramuscular injection of 0·25 ml. of a 15 hour glucose broth culture in 2 guinea-pigs resulted in death in 8 hours and approximately 12 hours respectively, with typical post mortem signs of *Cl. welchii* infection. The organism was recovered from the blood and most body organs. Guinea-pigs which had been actively immunised in pairs with toxoid prepared from *Cl. welchii* types A, B, C, D, E and the test organism were challenged intramuscularly in the thigh of the uninjected side 21 days after the third vaccinating dose with 0·25 ml. of a 15 hour broth culture. Two unvaccinated controls and those animals vaccinated with alum precipitated toxoid of types A, D and E all died within 24 hours. Complete protection was afforded by vaccination with toxoid preparations from types B and C, and the homologous test organism, though on post mortem examination 6 days later it was possible to recover the organism from the injection site of one animal vaccinated with group B toxoid in the absence of any sign of disease.

DISCUSSION

The organism isolated from the small intestine of chickens dying with lesions of necrosis of the small intestine and degeneration of the body organs caused by toxaemia had the typical morphological and biochemical characteristics of *Clostridium welchii*.

One departure from the normal findings was the unusual heat resistance of the spores obtained from culture. Laboratory induced sporulation normally results in spores that are very susceptible to heating at 100°C. Headlee (1931) found that spores were destroyed in a few minutes at 100°C. Zeissler and Rassfeld-Sternberg (1949) reported that spores of *Cl. welchii* types A, B, C, D and E were all destroyed in 15 mins. at 100°C, and Brooks, Sterne and Warrack (1957) confirmed their destruction at that temperature within 1 hour. Apart from the heat resistance of certain atypical pathogenic type A strains (Hobbs, Smith, Oakley, Warrack and Cruickshank, 1953; Brooks *et al.*, 1957), the only spores of *Cl. welchii* regularly resistant to a temperature of 100°C for 1 to 4 hours are those of type F (Zeissler and Rassfeld-Sternberg, 1949; Diekmann, 1949; Brooks *et al.* 1957). In the experiments described in this paper the spores of the test organism resisted heating at 100°C for 2 hours under conditions that quickly killed one strain each of types A, B, C, D and E.

The pattern of haemolysis on sheep blood agar plates, the ability of the toxin elaborated by the organism to lyse suspensions of rabbit and sheep cells, and the demonstration of lecithinase activity on egg yolk and human serum substrates provide incontrovertible evidence of the presence of α toxin. The lecithinase activity was demonstrated by Macfarlane *et al.* (1941) to be due solely to α toxin, and in these experiments the haemolysin activity could be inhibited by neutralisation with α antitoxin. Finally the test organism produced a precipitin reaction in the gel diffusion tests against α antitoxin which

corresponded with that produced by a known type A strain. In all these tests however the α toxin component appeared to be of low concentration.

The gel diffusion tests of the Elek (1949) pattern, culturing the test organism and an organism of known group in parallel lines at right angles to an antitoxin barrier, provided evidence that the antitoxin was potent and capable of precipitating with toxins formed by the homologous type strain of organism. Examination of the precipitin patterns of the known and the test organism for concurrence, the 'looping' described by Elek, also provided evidence of the antigenic identity of the components. The results of the tests on the unknown organism demonstrated a weak α component against anti-A antitoxin, and a strong β component when tested by anti B and anti C antitoxins. No precipitation occurred in the presence of the anti D nor anti E antitoxins. The precipitin pattern obtained by the homologous organisms in the presence of their antitoxic sera resemble those obtained by Oakley and Fulthorpe (1953) using a double diffusion technique in agar columns. They obtained single lines with types B, C and D in the presence of the respective antitoxic sera, and two bands in the A anti-A toxin system. In the experiments described above, an irregular second and third band was found in the A system, but there was one point of difference in that a secondary fainter band tended to appear in the B anti-B toxin system. The failure of this second precipitin line to appear in the B system of Oakley and Fulthorpe (1953) may be due to their selection of a type B organism which had lost its power to produce ε toxin. The strain of type B used in the above experiment retained this property, as in a control test it would give a faint reaction to an anti-D antitoxin concurrent with that produced by a type D strain. Other irregular faint precipitin patterns were found on some of the plates and were of uncertain significance. A greater complexity of patterns has been described in the detailed work of Elek (1949) and Orlans and Jones (1958), but the tests described here were adequate to demonstrate the presence or absence of the major toxic components.

An unexpected characteristic of the organism isolated from the disease in the fowl was its high titre of deoxyribonuclease activity. This was present in the fresh toxin in a dilution of 1/1024 and 1/2048. Warrack *et al.* (1951) reported that there was some difficulty in obtaining deoxyribonuclease from *Cl. welchii*, and that their *Cl. septicum* filtrates had about 250 standard indicating doses/ml. In these experiments on the test *Cl. welchii* there was no difficulty in demonstrating the presence of this enzyme in fresh toxin preparations, and using their criteria of 50 per cent. destruction of the leucocytes as the indicator of the titration end point, this toxin was more potent than that demonstrated in *Cl. septicum* filtrates. The failure of the commercial antitoxin sera to neutralise its activity is probably an indication of the absence of the specific antibody. Until this

toxin has been purified and tested by animal inoculation it cannot be assumed that it is the cause of the widespread lysis of nuclei in the intestine and liver, and the destruction of the leucocytes in the natural and experimental disease in the fowl, but such activity could theoretically produce these effects in vivo.

The animal inoculation tests demonstrating that the toxin was lethal to mice and dermonecrotic in guinea-pigs showed that it had properties in common with known *Cl. welchii* toxins. This toxic activity could be neutralised by previous addition of anti-β antitoxin prepared from either type B or type C organisms. Moreover, guinea-pigs could be actively immunised by type B and type C toxoids to give complete protection from an intramuscular injection of a viable broth culture of the test organism, and no such protection was afforded by toxoids prepared from type A, D or E organisms which do not produce the β toxin. Treatment of the toxin with trypsin after the manner of Bosworth and Glover (1934-5) did not increase its toxic properties.

A comparison of the characteristics of this strain of *Cl. welchii* isolated from the fowl, and the criteria used in classifying the various types of *Cl. welchii* described by Oakley and Warrack (1953) and Brooks *et al.* (1957) indicates that it belongs to type F, though its characteristics are not identical with those descriptions.

The test organism produces α toxin regularly though in fairly low concentration, as was demonstrated in all of the 20 strains of type F examined by Brooks and her colleagues. It also elaborated β toxin as was found in 17 of Brooks's strains, and this toxin was the component responsible for the death in mice as it could be neutralised by β antitoxin. The neutralisation of the mouse lethal toxin by β antitoxin in filtrates obtained from group F strains has been reported by Oakley (1949).

The presence of λ toxin described as occurring in type F filtrates (Oakley, 1949; Oakley and Warrack, 1953) was not observed in the chicken strain. Neither was the lysis of ox or horse erythrocytes not due to α, θ or δ toxins described in 15 of the 20 strains examined by Brooks *et al* (1957). There was no doubt of the presence of the v toxin (deoxyribonuclease) in the test strain, and it occurred in all 20 of Brooks's strains.

Another point of similarity was the resistance of the test organism to a temperature of 100°C which has been described by several workers as characteristic of type F, e.g. all 20 of Brooks's strains resisted heating for 1 hour, and 8 of them for 3 hours.

There is therefore evidence for regarding this *Cl. welchii* isolated from these cases in chickens as being a strain of type F, and there is the further tentative indication that the gross necrotic sloughing of the mucosa of the small intestine in the affected birds resembles those cases described in man and attributed to type F infection. However, the variability of *Cl. welchii* organisms is well known (McGaughey, 1933; Henderson, 1940; Hobbs *et al.*, 1953; Oakley

and Warrack, 1953; Brooks *et al.* 1957) so that identification of certain strains of organisms may be difficult, as both the somatic antigens and soluble toxins may differ from the criteria used to identify the known types. Thus mutability may also be a temptation to introduce new types into the present classification and Brooks *et al.* (1957) suggest that type F is an aberrant group, and should be regarded as a subgroup of type C.

CONCLUSIONS

The strain of *Clostridium welchii* isolated from cases of necrotic enteritis in the fowl was demonstrated to have many of characteristics of *Cl. welchii* type F. It elaborated α, β and ν toxins and the spores resisted 100°C for 2 hours. The lethal properties of the toxin for the mouse and chicken, and the dermonecrotic activity for the guinea-pig, could be neutralised by β antitoxin. Toxin obtained from cultures of the organism incubated for 5 to 10 hours have a high titre of deoxyribonuclease activity (ν toxin) which may be responsible for the karyolysis of the nuclei in the lesions of the natural and experimental disease.

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NECROTIC ENTERITIS IN THE FOWL
III. The Experimental Disease

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INTRODUCTION

In previous papers the histopathology of a fatal condition of young cockerels and the characters of a strain of *Clostridium welchii* isolated from the intestine of 27 of the affected birds has been described (Parish, 1961 a, b). The lesions observed in the naturally occurring condition were necrosis of areas of the small intestine and liver, and, in the chronic form, regression of all lymphoid and myeloid tissue. This paper presents the results of attempts to induce a similar disease in healthy birds.

MATERIALS AND METHODS

The birds were White Leghorn cockerels weighing 750 to 900 g, or commercially bred Light Sussex cockerels or pullets weighing 1650 to 1820 g. Five Rhode Island Red cockerels weighing between 5lb and $5\frac{1}{2}$ lb were used in an attempt to infect adult stock. The birds were fed a commercial balanced dry mash, with additional vitamins A and D in powder form weekly, and water was provided ad. lib.

To determine the packed cell volumes of the venous blood, samples were taken into a mixture of dry ammonium oxalate and potassium oxalate, or into dry heparin and gently agitated. Any samples containing clots were discarded. Two samples from each bird were centrifuged in Wintrobe haematocrit tubes for 30 mins at 3,000 r.p.m.

The strain of *Cl. welchii* obtained from natural cases of the disease and passaged 13 to 28 times on sheep blood agar or Robertson's meat medium, was used. In the first experiment the birds were dosed orally with 1 ml. of a 4 day culture, grown in the medium of Robertson and Keppie (1941), given either as one dose, or as 1 ml./day for 7 consecutive days. In the second experiment the birds were given 1/4 gr. of opium, 1·5 g. of calcium carbonate and 0·5 g. of sodium bicarbonate in the form of a thick suspension orally into the crop for 2 consecutive days, and were also dosed with 1 ml. of a four day broth culture of *Cl. welchii* 4 hours later on the second day. In the third experiment the birds received the same treatment, but the administration of the opium mineral salt paste was continued for a further 5 days.

Bacteriological examination of the faeces from the rectum of all experimental birds was carried out before and every 2nd or 3rd day after dosing. The material was shaken up at a dilution of 1/10 in phosphate buffered saline with sterile glass beads and about 0·05 ml plated out on sheep blood agar, 2 plates per sample, incubated anaerobically at 37°C for 24 hours and then left at room temperature for a further 12 hours. If a profuse growth of anaerobic organisms was obtained, the faecal sample was

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boiled for 15 min. in a water bath and 2 more plates inoculated to isolate the more heat resistant *Cl. welchii*. Any haemolytic organisms resembling *Cl. welchii* or a selection thereof if numerous, were subcultured onto sheep serum agar ditch plates to confirm their identity. The ditch plates were made of 10 per cent papain digest broth agar plus 10 per cent normal sheep serum as described by Bullen, Scarisbrick and Maddock (1953) and a ditch cut across the centre of the plate containing 2·0 ml of 4 per cent agar plus 0·6 ml. of *Cl. welchii* type C antitoxin containing 730 units anti-β/ml. The colonies to be tested were streaked at right angles to the ditch 12 colonies being tested on each plate. The plates were incubated anaerobically for 48 hours at 37°C and left at room temperature for a variable period.

A detailed post mortem examination was made of all birds dying of the experimental disease, or survivors when killed one month later. Histological examination of sections of the intestine, the liver, spleen and kidneys, and the bone marrow of affected birds, together with aerobic and anaerobic incubation of bacteriological media was carried out as described in the previous paper (Parish, 1961a). Contents of the small intestine of birds affected by the acute form were extracted in phosphate buffered saline 1:4 v/v, centrifuged at 3000 r.p.m. for 1 hour and the middle layer filtered through a 0·4 μ gradocol membrane. The fresh extract was injected intravenously in 0·2 ml. volumes into mice weighing about 20 g. Neutralization tests were carried out on the extracts in 0·4 ml. volumes with the *Cl. welchii* type C antitoxin at 37°C for 30 min. and 2 ml. amounts injected into mice. A control test was performed with inactivated normal horse serum diluted 1:4 in place of the antitoxin.

RESULTS

Single Dose Test Organism

Five birds dosed with a single broth culture of the test strain of *Cl. welchii* showed no clinical ill effects, and made the same gain on weekly weighing as uninoculated controls. One bird only excreted the organism on one occasion, when it was recovered in the faeces in small numbers 28 hours after dosing. Blood samples taken on the 2nd, 4th, 10th and 17th days after inoculation had the same packed cell volumes of red and white cells as the controls, and when the birds were killed on the 28th day histological examination of the body organs showed no characteristic lesions and no *Cl. welchii* elaborating β toxin was found in the contents of the small intestine or caeca.

Multiple Doses Test Organism

Eight birds dosed with 1 ml. of whole broth culture of the organisms daily for 7 days showed no significant signs of disease. Weight gains remained within the same range as the 13 controls, and the packed cell volumes remained within the normal range, though slight fluctuations were observed on consecutive tests taken at the same times as in the first group. On the 4th day after dosing, the faeces of two of the birds became fluid and remained so for the next 3 days. Bacteriological examination of faeces from the rectum of these birds showed small numbers of *Cl. welchii* elaborating β toxin

in 2 birds on the 2nd day after dosing, in small to moderate numbers in 5 birds on the 5th day, in 3 birds on the 7th day when dosing was discontinued, in only one bird on the 10th day and in none thereafter.

Two of the birds were killed on the 10th day, but apart from diffuse areas of necrosis in the crop and a slight increase in the number of lymphocytes in the blood vessels of the liver, no abnormal changes were observed: it was not possible to isolate the test strain of *Cl. welchii*. The remaining 6 birds killed on the 28th day showed no significant abnormality, and the test organism could not be recovered from the intestine.

Single Dose Test Organism with Opium

Twelve birds received a thick suspension of chalk, sodium bicarbonate and opium for 2 days, and on the second day a single dose of the whole broth culture of *Cl. welchii* was administered. All the birds lost weight in the week that they were dosed, but 9 of them returned to normal the following week. The remaining 3 continued to lose weight: one died on the 8th day, and the other 2 on the 11th and 12th days in a grossly emaciated condition.

The 9 survivors showed no significant change in the red or white packed blood cell volumes on the 3rd, 7th and 10th days after administration of the organisms. Bacteriological examination of faeces from the rectum on the 2nd and 5th day showed that all 9 were excreting the organism in small numbers, but only 5 continued to excrete the organism on the 8th day and one bird on the 10th. When these birds were killed on the 28th day no histopathological abnormality was observed and the strain of *Cl. welchii* could not be isolated from the intestine. The packed blood cell volume estimations on the 3 birds that died showed a reduction in the number of leucocytes on the third day and on the 7th day a very low haematocrit reading was obtained for the leucocyte-thrombocyte fraction. When this fraction was removed, and stained smears prepared for microscopical examination, few leucocytes were found to be present. The results of these tests determining the packed cell volumes are presented in Table 1.

The 3 fatal cases were in a severely emaciated condition, with a corresponding loss in weight of the liver and spleen; the data of these weights compared with those of the unaffected birds are presented in Table 2. On post mortem examination of these birds, the livers were small and congested with a few diffuse pale areas on the surface measuring about 1.5 cm on the long axis, but they were not easy to see. The spleens were small, firm and a pale grey in colour. In each bird the small intestine was affected from the jejunum to the commencement of the ileum. These affected areas measured from 8·5 to 11 cm. in length and consisted of a dilated portion of the intestine with thickened walls, and a pale, bile stained mucosa. There were little or no contents in the small intestine and the affected

TABLE 1

BLOOD EXAMINATIONS

Condition of bird	*Number of birds*	*Total packed cell volume %*		*Leucocyte—thrombocyte fraction %*	
		Average	*Variation*	*Average*	*Variation*
Control, undosed	13	28·2	27·3 – 30·0	2·2	1·9 – 2·8
Dosed, unaffected	9	27·8	26·1 – 29·4	2·3	1·7 – 3·0
Dosed, affected	3	20·5	18·6 – 23·3	0·7	0·3 – 1·1

Haematocrit readings of blood samples taken from the chickens on the 7th day after oral administration of the culture of *Cl. welchii*. Two tests were carried out on the blood of each bird.

portion contained a bile stained mucoid deposit that was not adherent to the mucosa. The remainder of the intestine was pale and flabby. The bone marrow had a pale pink colour and a dry texture. The other changes in the body were probably consequent on the emaciated condition; the heart was pale with a small unmeasured, volume of serous fluid in the pericardium, and the musculature of the legs was also pale and oedematous.

The histological changes observed in these birds closely resembled those described in the chronically affected birds dying of the natural disease. The affected parts of the intestine were slightly congested and oedematous and several of the blood vessels of the submucosa were occluded by thrombi. There was degeneration of the epithelium of the villi extending down to the full depth of the mucosa including the glandular tubules, and the stroma persisted as necrotic anucleate strands. There were no leucocytes present in any of the affected areas. The livers were congested, though some of the smaller blood vessels were occluded by thrombi. These thrombi contained a few

TABLE 2

BODY WEIGHTS

	Normal (total 9) 28 days after dosing		*Affected (total 3) 8th to 12th day after dosing*	
	Average	*Variation*	*Average*	*Variation*
Body weight	820	750 – 900	620	540 – 700
Weight of liver without gall bladder	21·5	16·8 – 24·2	13·6	12·7 – 14·9
Weight of spleen	0·72	0·37 – 1·1	0·18	0·15 – 0·21

The weights in grammes of the carcase, liver and spleen of young White Leghorn cockerels. The 3 affected birds died on the 8th, 11th and 12th days after receiving the culture of *Cl. welchii*. The normal birds were those which showed no clinical ill effects after dosing with the bacterial culture, and were killed by intravenous Nembutal 28 days later.

macrophages loaded with pigment. There were also irregular droplets of nuclear material which could be stained by the Feulgen technique and was susceptible to extraction by deoxyribonuclease. Beneath the capsule and surrounding a few of the blood vessels were zones of degeneration leading to necrosis, and areas of characteristic nuclear karyolysis as described in the natural disease (Parish 1961a). There was severe degeneration of the lymphoid tissue of the spleen and myeloid tissue of the bone marrow and no mature leucocytes could be found in any preparation. In one spleen there was a small necrotic area on the surface extending into the substance of the organ.

Bacteriological examination of the carcase resulted in a moderate growth of *Cl. welchii* from the affected part of the small intestine, but none from the liver, gall bladder, spleen, heart blood, kidney or bone marrow. Two of the birds had a sparse growth of *Escherichia coli* from the liver and bile, and one plate had a few colonies of Proteus species in material from the kidney.

Prolonged Administration of Opium (W.L.)

Five well matched young White Leghorn cockerels from the same hatching and weighing between 800 g. and 860 g. were selected for an experiment to determine the effects of a more prolonged disturbance of normal intestinal function. They were dosed orally with the mineral salt, opium suspension daily for 7 days, and also received 1 ml. of a whole broth culture of *Cl. welchii* on the 2nd day. Two of the birds showed signs of disease on the 2nd day after administration of the cultures, with ruffled feathers, drooping head and wings and passing dark fluid faeces. One died on the 3rd day, and the other was killed by intravenous Nembutal when in extremis on the same day to ensure that the post mortem examination could be performed on the fresh carcase.

Both these birds presented the post mortem picture of the acute disease. In one there was a single portion of the ileum, 8 cm. long and ending 16 cm. anterior to the site of origin of the blind caeca. The other had two more diffuse affected areas, one in the mid portion of the intestine and the other at the commencement of the ileum. The areas contained a core of debris in the lumen stained with blood and bile. Smears of the contents of the lumen stained by methylene blue or Gram revealed a mass of necrotic epithelial cells, intact erythrocytes and many Gram positive, rod-form bacteria, in some of which it was possible to observe spores. On histological examination the affected areas were composed of a necrotic mucosa where a core of degenerating epithelial cells, partly fused together, was attached to or in the process of separating from the submucosa. There was slight haemorrhage and extensive oedema in the surrounding tissues, scattered fibroblasts in the periphery and a few lymphocytes and heterophils. The livers of both birds were congested, and a few blood vessels contained thrombi and Feulgen positive nuclear material in strands and droplets. These vessels were surrounded by

small areas of necrosis and other necrotic areas containing degenerating cells with karyolysis of the nuclei were found under the capsule. The spleens of both birds were severely congested, and there was a variable degeneration of the lymphoid follicles where some of the cells in the centre of the follicles had vacuolated nuclei and pale unevenly staining cytoplasm. The kidneys were also slightly congested, and contained a few lymphocytes and heterophils.

On bacteriological examination, *Cl. welchii* was obtained in moderate growth from the affected areas of the small intestine and from faecal material obtained from the rectum. This organism could not be isolated from the contents of the caeca, or from the other body organs.

A saline extract of the contents of the small intestine of these two birds, prepared on the day of death and injected intravenously into mice, killed them within 15 minutes. Incubation of these extracts with *Cl. welchii* type C antitoxin inhibited the mouse lethal property of the extract, but when incubated with normal horse serum diluted 1:4 the mixture killed mice.

Prolonged Administration of Opium (*R.I.R.*)

Five Rhode Island Red cockerels just over 5 months of age and weighing between 5 and 5½lbs. were dosed in the same manner as the birds in part one of the 3rd experiment. All the birds had lost weight at the end of the week until the average weight was 4lb. 9oz. In the 3 succeeding weeks there was an average gain in weight of 3 oz. the first week, 5 oz in the second and 4 oz. in the third.Blood samples taken before the experiment commenced and on the 5th, 9th and 14th days after the birds had received the bacterial cultures had a lower total packed cell volume on the 5th and 9th days, but the average decrease was only 2·3 per cent and was almost within the limits of experimental error when readings of the 2 samples obtained from each bird were compared. *Cl. welchii* was obtained from the faeces on the 5th and 9th day after dosing from all birds, but only from 2 in small numbers on the 14th day, and none thereafter. When the birds were killed on the 28th day no histopathological abnormality was found, and no *Cl. welchii* capable of producing β toxin were isolated from the intestine.

DISCUSSION

The strain of *Cl. welchii* isolated from birds dying as a result of natural infection and maintained on laboratory media for 13 to 28 passages did not prove very infective under experimental conditions. This may have been due to some loss in virulence of the organism, or to the natural condition being a sequel to some derangement of alimentary tract function. It is possible that both these factors were implicated, but the evidence suggests that some predisposing cause is necessary. No bird succumbed to infection when dosed with the organism passaged 13 times on laboratory media,

even after 7 consecutive daily doses, yet 5 of 22 birds developed the disease following dosing with the organism passaged on media 28 times after they had been pretreated with mineral salts and opium, though of these only 2 developed the acute disease. Age appeared to be a factor since 2 of 5 young birds developed the infection when not one of the 5 adults proved susceptible.

Difficulties have been encountered by several workers in attempts to induce infection by feeding cultures of *Cl. welchii*. Bullen and Batty (1957) have briefly reviewed the attempts to infect sheep with *Cl. welchii* type D. Bennetts (1932) Roberts (1938) and Harshfield, Cross and Hoerlein (1942) managed to infect sheep by feeding cultures together with certain other products, whereas using somewhat similar methods, their results could not be confirmed by Oxer (1932), Gill (1933), and Shaw, Muth and Seghetti (1939). In a series of critical investigations (Bullen, 1952; Bullen *et al.* 1953; Bullen and Scarisbrick, 1957; Bullen and Batty, 1957), some explanation for these variable results was obtained. It was found that many normal sheep tolerate the presence of *Cl. welchii* type D in the intestine, but infection may result from some derangement of the normal alimentary flora as caused by some sudden change in diet when considerable quantities of toxin may be produced.

These conditions appear to resemble those present in the *Cl. welchii* infection of the fowl. The disease resulting from the experimental infection had identical histopathological lesions in both the acute and chronic form to those encountered in the natural disease, with a similar reduction in the number of circulating leucocytes, and the strain of *Cl. welchii* and a mouse lethal β toxin could be isolated from the contents of the small intestine of birds affected by the acute form of the disease. Yet this infection was only produced when the cultures were given in conjunction with doses of mineral salts and opium. The natural outbreak of the disease occurred within five days of the birds being obtained from an outside source (Parish, 1961a) which involved a change of environment and diet, though other birds of similar age reared on the premises remained unaffected under the same conditions.

The enterotoxaemia of fowls and the strain of *Cl. welchii* isolated from the affected birds were considered to be similar to the disease 'enteritis necroticans' in man and the *Cl. welchii* type F associated with it, (Parish 1961 a, b). This condition in man also appears to be related to some alimentary disturbance, such as overeating (Jeckeln, 1947; Hain, 1949). Siegmund (1948) and Schutz (1949) though suggesting several possible predisposing causes, stress the probable importance of disease or physiological dysfunction of the alimentary tract.

On theoretical grounds any species of Clostridium capable of multiplying in large numbers in the intestine of the fowl and elaborating necrotizing and deoxyribonuclease toxins would be capable of causing a similar disease, but it is possible that this strain of

Cl. welchii has particular properties which enable it to establish itself in the intestine under certain conditions. Koslowski, Schneider and Heise (1951), investigating the properties of *Cl. welchii* type F obtained from necrotic enteritis of man, found that at a pH between 5·1 and 8·0 it produced high concentrations of histamine from histidine, which may be an important factor in the causation of the necrosis. This was not found to occur in four other species of Clostridia investigated.

Variation in virulence of any type of *Cl. welchii* may also be a complicating factor. Hain (1949) observed that *Cl. welchii* type F obtained from the faeces of normal human beings was less pathogenic on intramuscular injection into guinea pigs than organisms of the same type isolated from cases of 'enteritis necroticans'. A similar phenomenon was encountered in experiments testing the pathogenicity of atypical heat resistant strains of *Cl. welchii* type A associated with food poisoning in man. In their experiments Hobbs, Smith, Oakley, Warrack and Cruickshank (1953) demonstrated that volunteers who ingested cultures of these strains of *Cl. welchii* type A were mildly affected, though Dische and Elek (1957) found that one strain produced symptoms in most of the volunteers, a second strain gave indefinite results and a third appeared inactive. They suggested that the virulence of the strain may have deteriorated.

No tests were carried out to attempt to determine loss of virulence of the strain of *Cl. welchii* obtained from the fowl, but the evidence obtained by other workers suggests that the onset of disease may depend as much on the virulence of the strain of organisms present in the intestine as on the predisposing conditions of alimentary dysfunction. Little is known of the incidence of Clostridial organisms in the intestine of the fowl.

CONCLUSIONS

A strain of *Clostridium welchii* obtained from the intestine of fowls affected by necrotic enteritis and passaged 13 to 28 times on laboratory media was used to induce experimental infection in healthy birds. Single, or repeated daily oral doses of 4 day whole broth cultures failed to cause infection, but cultures given at the same time as a mixture of calcium carbonate, sodium bicarbonate and opium produced disease in 5 of 22 birds.

The histopathology of the lesions in the experimentally affected birds resembled that of both the acute and chronic forms of the natural disease, and the organism could be recovered from the small intestine. It is suggested that some dysfunction of the alimentary tract is a necessary predisposing cause of infection.

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ADAPTATION OF THE INFECTIOUS AGENT OF TICK-BORNE FEVER TO GUINEA-PIGS AND MICE

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INTRODUCTION

The study of tick-borne fever has been hindered by the failure to reproduce the disease in laboratory animals.

Hitherto the condition has been transmissible only to the natural hosts, sheep and cattle, and to the goat (MacLeod and Gordon, 1933; Hudson, 1950; Foggie, 1951) with a consequent limitation of the scope of immunological experiments. To quote Hudson: "Immunity in tick-borne fever presents an intriguing problem and it is unfortunate that no small laboratory animal has yet been found susceptible so that this problem can receive the attention it deserves". In his study of *Cytoecetes microti,* a parasite of the vole, *Microtus pennsylvanicus,* which closely resembles the tick-borne fever organism, Tyzzer (1938) used splenectomy to stimulate patent infections in recovered animals. Similarly, splenectomy provoked a recurrence of parasitaemia in a sheep which had recovered from an earlier attack of tick-borne fever (Foggie, 1951).

These experiments suggested that the spleen played a part in the suppression of the infection and a further attempt was made to adapt the tick-borne fever organism to small animals, using if necessary splenectomised subjects. As the classification of the organism is still obscure it is, for convenience, referred to throughout the paper as 'the virus'.

METHODS

Animals. Rabbits, guinea-pigs and white Swiss mice from the Moredun closed colonies of these species were used throughout the experiments. All sheep used to test infectivity were born and reared at the Institute, which is tick-free.

Splenectomy operation on guinea-pigs. The evening before the operation green food was withheld from the guinea-pigs, leaving them only grass-nuts and hay, and the hair was removed from the left flank with mechanical hair clippers. Before operation, deep narcosis was induced by the intraperitoneal inoculation of 0.1 ml. of a 50 per cent. dilution of nembutal in normal saline per 100 g. body weight. Anaesthesia was maintained during the operation with ether. The spleen was removed through a flank incision after separate ligation of the dorsal and ventral blood vessels with No. 3 silk. The peritoneum and muscle layers were closed with one continuous suture of No. 0 silk and the skin incision was closed with interrupted nylon sutures. Although aseptic precautions were taken, in earlier operations abscesses developed at the operation site in one or two of the guinea-pigs. This trouble was overcome by sprinkling a synthetic antibacterial powder* into the operation wound before suturing. The mortality rate, including anaesthetic accidents, in about 300 operations has been less than 10 per cent.

* Decamethylene-Bis-(4-aminoquinaldinium chloride) 'Dequadin', Allen & Hanburys.

Splenectomy operation in mice. The operation in mice was performed under intravenous nembutal anaesthesia, supplemented with ether if necessary. A dose of 0.5 ml. of a 1/20 dilution of nembutal in saline usually produced surgical anaesthesia in a 20 gramme mouse. About 600 mice have been splenectomised; approximately 10 per cent. died before recovering from the anaesthetic but deaths in the survivors were rare.

Collection of infected blood. Blood for transmission of the infection was collected into Hanks fluid containing 500 units of heparin per ml., in the proportion of four parts blood to one part heparin solution. Sheep were bled from the jugular vein, rabbits by puncturing an ear vein and guinea-pigs by cardiac puncture. In most of the work on mice blood was collected by the following technique: The mice were given an intravenous injection of 0.25 ml. of dextran sulphate (Dexulate) which prevented clotting of the blood. They were then anaesthetised with ether, decapitated and suspended over a funnel through which the blood ran into a collecting bottle. A plentiful supply of blood (about 1 ml. from each mouse) could be obtained by this method, but it had the great disadvantage that the course of the infection could no longer be followed in the donor mice and there was a danger of losing the strain if the recipient mice failed to react. In later experiments blood for transmission was obtained from mice by cardiac puncture. Using a No. 19 needle, blood was drawn into a 1 ml. syringe charged with 0.1 ml. of heparin solution. About 0.25 to 0.5 ml. of blood was taken from each mouse. From 20 to 30 per cent. of the mice died from cardiac haemorrhage following this procedure but survivors appeared to be normal in 24 hours. This technique had the additional advantage that the blood could be collected aseptically.

Transmission of infection. Intravenous inoculation of infected blood was used to transmit infection to sheep, rabbits and mice; sheep and rabbits were given from 1 to 2 ml. of blood and mice 0.25 ml. of blood. Initially guinea-pigs were inoculated intraperitoneally with 3 to 5 ml. of blood. Later, when a normal guinea-pig adapted strain of the virus had been established, it was found that the prepatent period of the infection tended to be shorter following the intracardial inoculation of 1 ml. of infected blood and this technique was adopted thereafter.

Temperature recording. Rectal temperatures of rabbits, guinea-pigs and sheep were recorded daily. No attempt was made to record body temperatures of mice.

Examination of blood films. Thin films were made from blood obtained by puncturing an ear vein in rabbits, sheep and guinea-pigs and a caudal vein in mice. MacNeal's modification of Leishman's stain was used throughout the experiments. One hundred polymorphs (neutrophils and eosinophils) on each film were examined for the presence of inclusion bodies and the result was recorded as the percentage of these cells infected. Although the virus is occasionally seen in basophils it tends to be obscured by the granules and for this reason these cells were omitted from the infected cell counts. Blood films were examined from sheep when the body temperature exceeded 104°F, from rabbits and guinea-pigs daily from the 2nd or 3rd day after inoculation until the 14th to 21st day after inoculation, and from mice at intervals of 2 or 3 days for 3 weeks after inoculation.

RESULTS

Rabbits

A rabbit was inoculated with 1 ml. intravenously and 3 ml.

intraperitoneally of infected sheep blood. After 7 days a second rabbit was inoculated intravenously with 1 ml. of blood from the first rabbit and 2 more blind passages were made in this manner. No inclusion bodies were seen in blood films from these rabbits and no temperature reactions occurred. A sheep inoculated with blood from the last rabbit in the series failed to develop tick-borne fever.

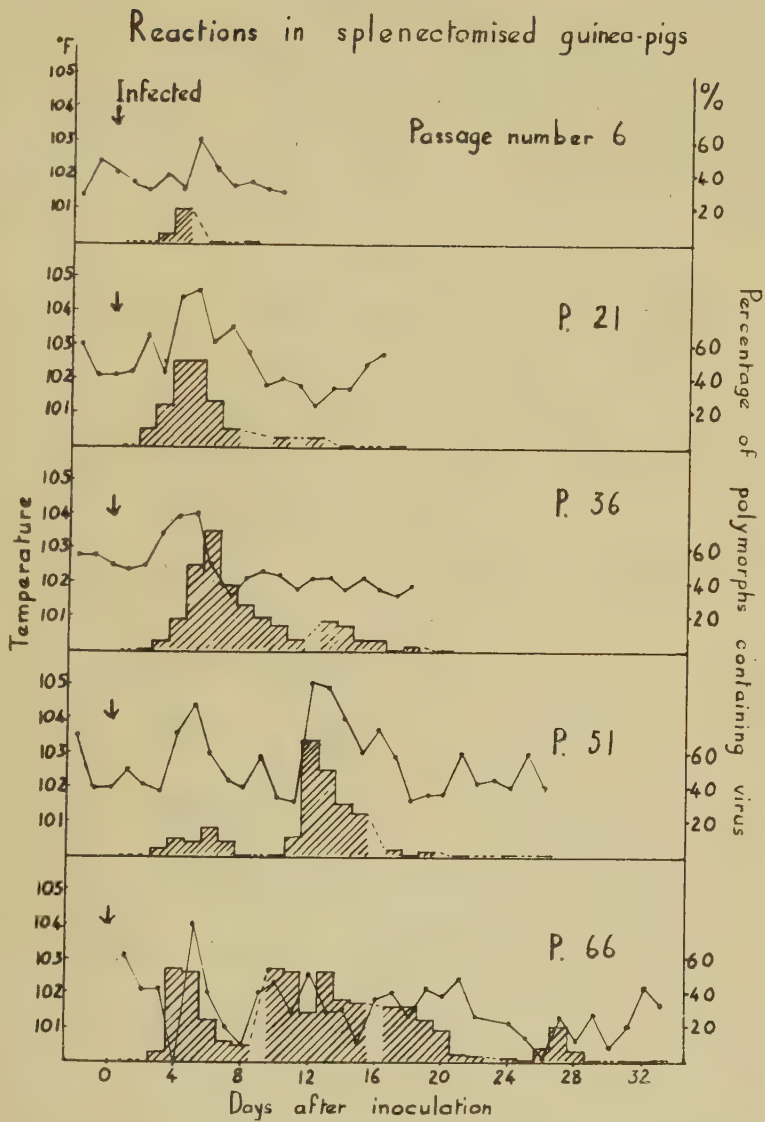
Guinea-pigs

Preliminary experiment on normal guinea-pigs. In a similar experiment to that on the rabbits, 2 normal guinea-pigs were inoculated intraperitoneally with infected sheep blood. Four blind passages in pairs of normal animals were made at weekly intervals without producing either temperature reactions or polymorph inclusions. Sheep inoculated with blood from the first and second passage guinea-pigs did not react.

Experiments on splenectomised guinea-pigs. Two splenectomised guinea-pigs were inoculated with blood from an infected sheep. A week after inoculation, blood from the guinea-pigs was inoculated into a sheep and another splenectomised guinea-pig. A typical tick-borne fever reaction (elevated temperature and numerous neutrophilic inclusion bodies) appeared in the sheep, but no virus was seen in the guinea-pig and its blood failed to infect a test sheep. On the 21st day after the primary inoculation, typical virus was seen in about 10 per cent. of the polymorphs in the blood of one of the two original splenectomised guinea-pigs. Blood from this animal was inoculated into another splenectomised guinea-pig in which virus appeared on the 4th, 5th and 6th days after inoculation. Thereafter the infection was maintained in serial passage, at intervals of about 10 days, in splenectomised guinea-pigs.

There was a gradual increase in the percentage of parasitised polymorphs and, as the series progressed, secondary and frequently tertiary crises occurred (Fig. 1). Although a temperature reaction was recorded in most of the infected animals the degree and duration of pyrexia was variable. Until the 33rd passage about 1 in every 4 animals failed to show any signs of infection; thereafter the infection rate has been 100 per cent. After the 67th passage the interval between transmissions was increased to 21 days without any apparent loss in virulence of the infection which is now in the 84th passage. In animals in later passages there was some loss of appetite and listlessness at the temperature crises, but all of the few deaths which have occurred could be related to causes other than tick-borne fever. Recovered animals appeared to be quite healthy and rapidly regained any weight lost. The uncomplicated disease in sheep follows a similar course, but secondary crises are less frequent and virus usually disappears from the polymorphs in 5 to 10 days after which there is a profound neutropaenia lasting for several days. We have been unable to detect any marked drop in the neutrophil count following the infection in splenectomised guinea-pigs. Although this

Fig. 1

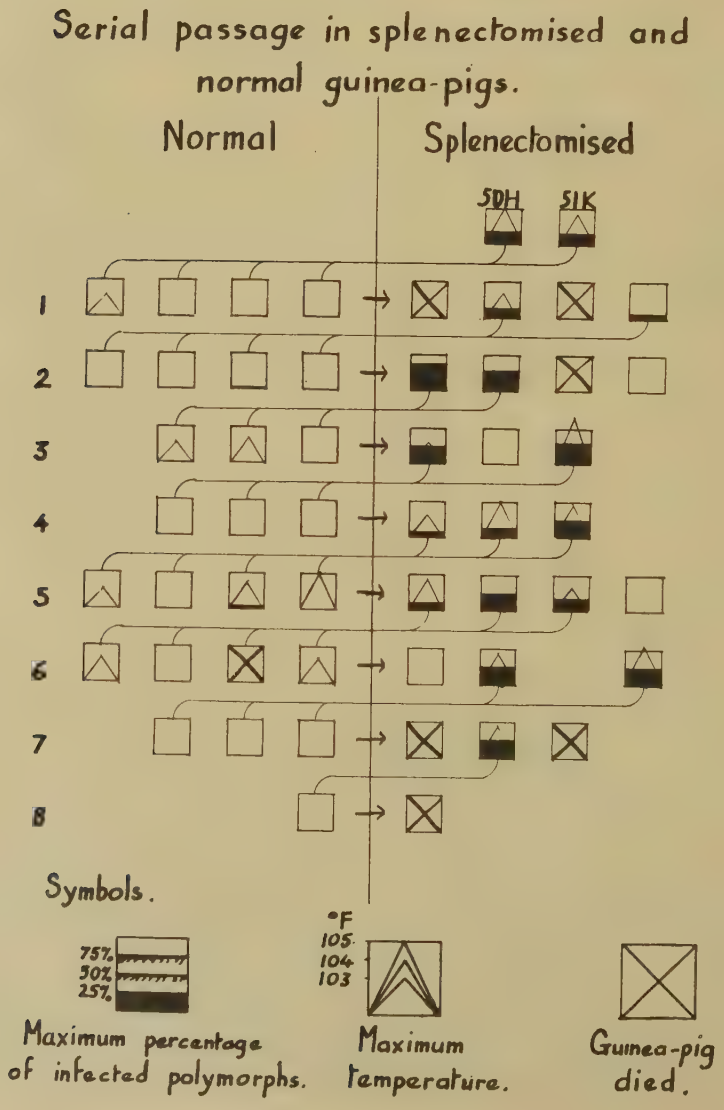


strain of virus appeared to be completely adapted to the splenectomised guinea-pig the elaboration of the operation precluded its use in experiments requiring more than a few animals and further steps were taken to adapt the strain to normal animals.

Adaptation of the virus to normal guinea-pigs. Starting with blood from an infected 7th passage splenectomised guinea-pig, blind serial passages were made in eight pairs of normal guinea-pigs. A few inclusion bodies were seen in blood films from both the 6th passage

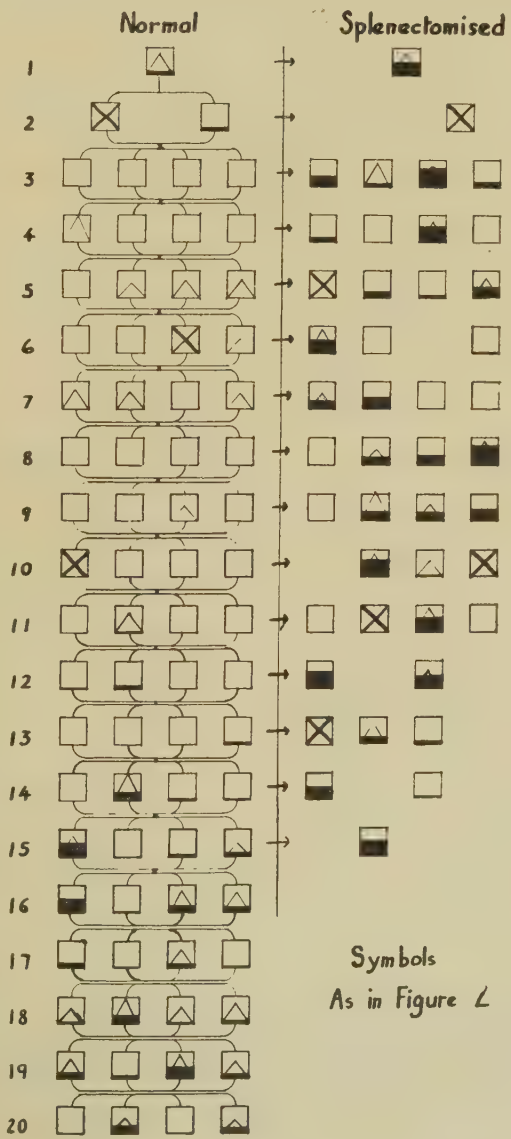
animals, but virus failed to reappear in 7th and 8th passage animals and the line was discontinued. Alternate passage in normal and splenectomised guinea-pigs was carried out by inoculating normal animals with infected blood from splenectomised animals (50th and 51st passage) and performing splenectomy one week later. Blood from animals in which the infection became apparent after the operation was inoculated into further normal animals and the process repeated. This experiment is shown diagramatically in Fig. 2.

Fig. 2



Virus was seen in one normal guinea-pig at the 5th passage, but did not reappear in normal animals in 3 further transmissions. Blood from the reacting normal guinea-pig (5th passage—Fig. 2) was inoculated into 2 guinea-pigs and passed in series in normal animals each week as is shown in Fig. 3. Virus was seen in one of the 2 second passage animals, but did not reappear for a further 8 passages. The

Fig. 3



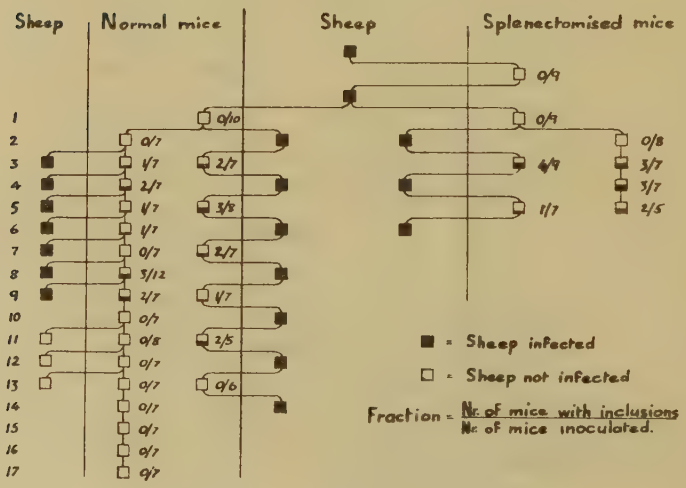
presence of infection was, however, confirmed by splenectomy of the donor animals 7 days after each transmission, when the majority gave good reactions. From the 111th passage onwards virus was seen in a gradually increasing proportion of the normal guinea-pigs and splenectomy after transmission was discontinued. The strain is now in the 63rd passage, but does not yet appear to be fully adapted to the normal guinea-pig. As is shown in Table 1 numerous infected cells were seen in approximately half the animals used in passages 47 to 59. Although a temperature reaction occurred in the majority of animals in which infected cells were numerous this was not consistent.

TABLE 1

PROPORTION OF STRONG AND WEAK REACTIONS IN NORMAL GUINEA-PIGS IN PASSAGES 47-59.

Maximum percentage of infected cells during reaction	*Number of animals*
over 10%	15
less than 10%	16
no virus seen	1
Total number of animals	32

Fig. 4

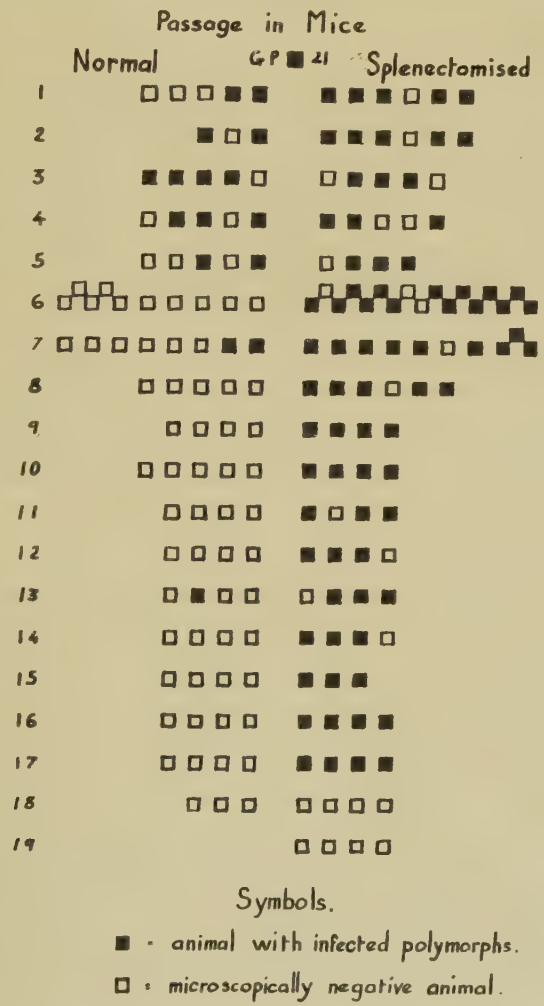


Mice

Adaptation to splenectomised mice. The first experiment is shown diagramatically in Fig. 4. Splenectomised mice were inoculated with infected sheep blood. No virus was seen in blood from the mice which were killed 7 days after inoculation, but a sheep inoculated with this mouse blood developed tick-borne fever. Groups of normal and

splenectomised mice were inoculated with blood from the sheep and from these groups mouse to mouse passage lines and mouse-sheep alternate passage lines were started. Virus was seen in a few animals in all the groups of mice at the 3rd passage. As the line in normal mice appeared to be established the line in splenectomised mice was discontinued after the 5th passage. The virus was carried success-

Fig. 5



fully through 9 passages in normal mice, but failed to appear in the 10th or subsequent passages and test sheep inoculated with mouse blood failed to react.

The second isolation of a strain of virus in mice is shown in Fig. 5. Blood from an infected splenectomised guinea-pig was inoculated

into normal and splenectomised mice. Virus was seen in the polymorphs of 2 out of 4 normal mice and 5 out of 6 splenectomised mice. The microscopically infected animals were killed and a pool of their blood was inoculated into further normal and splenectomised mice and the infection was passed in series. The interval between transmissions was 2 to 3 weeks. As is shown in the figure this strain appeared to become adapted to splenectomised mice and, with a few exceptions, ceased to produce visible infection in normal mice after the 5th passage. The virus suddenly disappeared at the 18th passage and was not recovered.

The isolation of another strain in splenectomised mice proved very difficult. After several failures, using both infected sheep blood and infected guinea-pig blood, a series of blind passages in groups of 5 splenectomised mice was started, the source of infection being blood from a splenectomised guinea-pig. At each transmission blood from the mice was inoculated into a susceptible sheep. All the sheep reacted indicating that the infection was still present, but the infection did not become patent in the mice until the 10th passage, after which cell inclusions were seen in increasing numbers of the animals in successive passages.

This strain is now in the 46th passage in splenectomised animals. In the last 15 passages 117 mice have been inoculated and in only one was no virus seen. The mean of the maximum percentage of infected polymorphs recorded in these mice was 10.51 per cent. ($\pm$ 5.72 per cent.). Infected cells were seen in most of the mice by the 3rd day after inoculation and continued to appear for from 2 to 3 weeks thereafter.

Passage in normal mice. A strain of virus was passed alternately in normal mice and sheep. At the 4th and 5th passages virus inclusions were seen in smears from some of the normal mice and at the 6th passage a direct mouse to mouse series was started. This strain was maintained for 24 passages, but the infection did not appear to increase in virulence for normal mice. Although there is no doubt that for the 12 months duration of the experiment virus multiplication was taking place, the virus was rarely detected in more than one or two of the 7 mice used in each passage and in these only one or two infected cells were seen. Sheep inoculated with mouse blood from the 5th, 6th, 10th, 12th, 15th and 18th passages all developed tick-borne fever. The strain was lost at the 24th passage. The infectivity of blood from this group of mice was tested in normal and splenectomised mice, a splenectomised guinea-pig and 2 susceptible sheep, with negative results. Attempts to recover the strain from surviving mice in earlier passages also failed.

Effect of Pretreatment with Cortisone and Nitrogen Mustard on the Susceptibility of Mice

At an early stage in the study of the infection in mice an experiment was performed to determine whether pretreatment with

cortisone or nitrogen mustard enhanced the susceptibility of mice to the infection.

Four groups were selected at random from a pool of 37 mice weighing 20 grammes ± 1 gramme. The mice in the first group were inoculated subcutaneously with a daily dose of 5 mg. cortisone for 5 days prior to infection. The mice in the second group were inoculated intravenously with 0.05 mg. nitrogen mustard (Foggie, 1957) 5 days before infection and the mice in the third group were splenectomised 4 days before infection. The remaining mice formed an untreated control group. On the 5th day the mice in each group were weighed, and total and differential white cell counts were made on 3 mice from each group. All the mice were then inoculated with blood from infected splenectomised mice (6th passage—Fig. 5). Blood films were examined at intervals for 12 days after inoculation, by which time the differential counts were within the normal range. The results are shown in Table 2. Thus, although cortisone reduced the lymphocyte count and nitrogen mustard the neutrophil count, and both agents produced a marked loss of weight, the pre-treated mice were no more susceptible to the infection than the untreated control mice. The splenectomised mice were, however, significantly more susceptible than the other groups.

TABLE 2

EFFECT OF PRETREATMENT WITH CORTISONE, NITROGEN MUSTARD AND OF SPLENECTOMY ON SUSCEPTIBILITY OF MICE

Pretreatment	*Day*	*At infection on day 5*			*Proportion of mice in which virus was seen*
		Mean weight	*Mean neutrophil count (3 mice)*	*Mean lymphocyte count (3 mice)*	
Cortisone	1–5	16.0 g.	3387/cmm	297/cmm	1/10
Nitrogen mustard	1	16.9 g.	219/cmm	1878/cmm	0/9
Splenectomy	2	17.8 g.	3983/cmm	7910/cmm	7/10
None	–	19.7 g.	1643/cmm	5990/cmm	2/8

Cross-infectivity of Laboratory Animal Adapted Strains

The 3 laboratory animal adapted strains viz. splenectomised guinea-pig strain (77th passage), normal guinea-pig strain (49th passage) and splenectomised mouse strain (41st passage) were tested for their ability to infect other animals. The results are shown diagramatically in Fig. 6.

Splenectomised guinea-pigs were susceptible to all 3 strains. Normal guinea-pigs were susceptible to the homologous strain (2 per cent. infected cells were seen on one occasion in one of the animals inoculated with the splenectomised guinea-pig strain). Splenectomised mice were susceptible to the homologous strain (2 per cent.

Fig. 6

Infectivity of laboratory-animal adapted strains of tick-borne fever virus
Animals inoculated
Strains
Splenectomised guinea-pig
Normal guinea-pig
Splenectomised mouse
Sheep
Infected polymorphs per cent.
100
50
0
Splenectomised guinea-pigs
Normal guinea-pigs
Splenectomised mice
Normal mice
Sheep
Days after inoculation
3 6 9 12
78Z
Y
X
D1
D2
50a
50b
A1
A2
45

infected cells were seen in one of 5 animals inoculated with the normal guinea-pig strain). Virus was seen in 5 out of 7 normal mice inoculated with the splenectomised mouse strain, but was absent in the other groups of normal mice. With the exception of one sheep inoculated with the normal guinea-pig strain in which the reaction was minimal, typical mild reactions were produced in sheep by all 3 strains. Histiograms of the reactions of 2 sheep infected with a sheep strain are included in the figure for comparison.

Appearance of the Virus in Guinea-pigs and Mouse Polymorphs

The appearance of the virus in guinea-pig blood is similar to that already described in sheep blood (Foggie, 1951). The large majority of infected cells contain small pleomorphic initial bodies which can only be identified by their blue-grey colour (Plate 1A). In heavily infected animals a proportion of the infected cells contain 'morulae' and fragmenting forms similar to those seen in sheep (Plates 1B and 2).

Although parasitised cells are less numerous in infected mice than in infected guinea-pigs, the virus inclusions are distinct and more easily recognised. The majority of infected cells contain multiple inclusion bodies which stain dark blue or even black and are frequently enclosed in, or associated with, a vacuole (Plates 3 and 4). Another characteristic of the infection in mice is the greater affinity of the virus for eosinophils. These may be the only cells infected even in animals with approximately normal differential counts.

In the course of the experiments a considerable number of infected guinea-pigs and mice were sacrificed and impression smears were examined from bone-marrow, spleen, liver, kidney and lung. Except for inclusions in mature polymorphs, nothing resembling tick-borne fever virus was seen in any of these tissues, either in animals with numerous infected blood cells or in animals in which the infection was not patent. Crush preparations of brain, examined for the presence of inclusions in the blood vessel intima such as occur in heartwater, showed no abnormality.

DISCUSSION

The inoculation of blood infected with tick-borne fever into an animal may be followed by one of three possible states:

(a) The virus may be destroyed and the animal remain uninfected.

(b) The virus may remain alive but fail to multiply sufficiently to be visible in the circulating polymorphs although the infection can be passed to other animals by the inoculation of blood. This condition was seen in the earlier passages in the normal guinea-pig experiment and in the second splenectomised mouse experiment. Although an occasional infected cell was seen in the normal mouse passage experiments the majority of the animals remained in this latent infection phase and stable adaptation to the normal mouse has not yet been achieved.

(c) The virus may multiply rapidly and infected cells become visible in the blood within 3 to 10 days of inoculation. This stage was

reached after one passage in the splenectomised guinea-pigs, but patent infection was only achieved after numerous blind passages in normal guinea-pigs and, although the strain now appears to be adapted, the reaction in a proportion of the animals is still minimal. In two of the experiments on splenectomised mice the virus adapted rapidly, but in others frequent blind passages were necessary before infected cells were seen.

In spite of numerous passages in small animals all 3 strains of virus adapted to laboratory animals were still infective for sheep in the cross-infection experiment. This finding contrasts with the difficulty experienced in infecting sheep with bovine strains of tick-borne fever and adds further weight to the suggestion that there may be specific differences between the bovine and ovine virus. (Foggie and Allison, 1960).

The following points may be made on the suitability of the laboratory animal adapted strains for further studies of the tick-borne fever organism. The splenectomised guinea-pig and splenectomised mouse strains produce easily diagnosed infection in 100 per cent. of homologous animals, but the necessity of using splenectomised animals restricts their usefulness. Although the numbers of parasitised cells in normal guinea-pigs, infected with the homologous strain, may not be so great as in splenectomised animals, it is rarely difficult to diagnose infection and any slight disadvantage is outweighed by the availability of large numbers of experimental animals. The unpredictable behaviour of normal mouse adapted strains renders them unsuitable for experimental use. Although it might have been possible to adapt the virus to splenectomised and normal rabbits, accommodation for stock of this species in this Institute is limited, and this aspect of the investigation was not pursued.

CONCLUSIONS

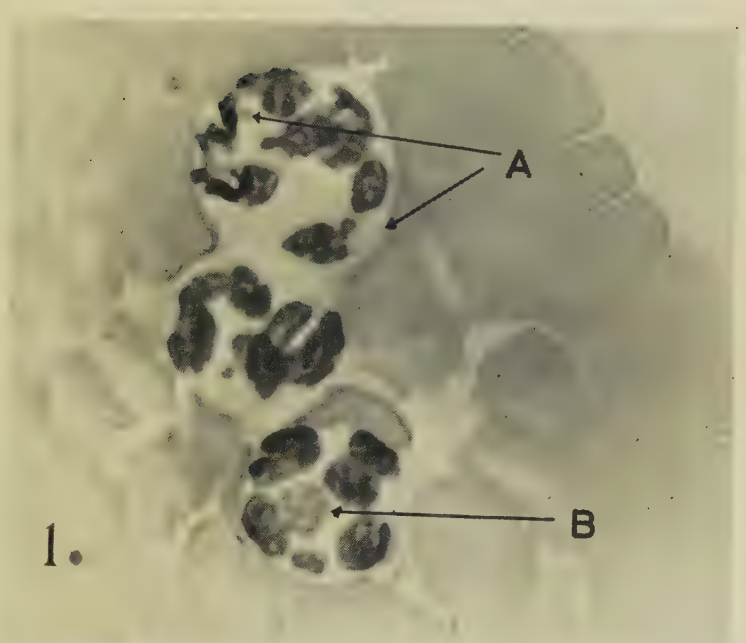
A strain of tick-borne fever virus was adapted to and has been maintained in splenectomised guinea-pigs for 80 passages. The disease in the splenectomised guinea-pig is similar to, but of longer duration than, the disease in sheep.

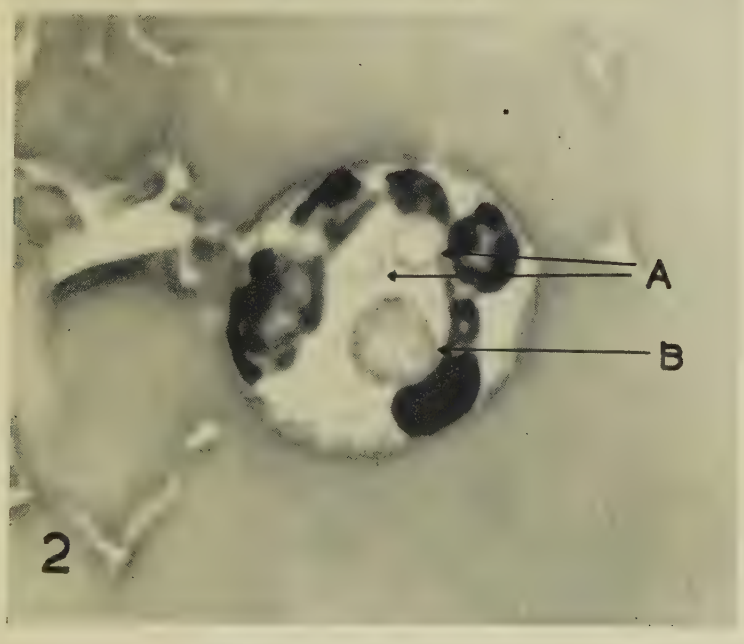
By alternate passage in normal and splenectomised animals, followed by blind passage in normal animals, the strain was adapted to normal guinea-pigs and has been maintained for 62 passages. Although in about half of the experimental animals the percentage of infected cells remained low and temperature reactions were not consistent, the strain appears to be stable.

A strain adapted to splenectomised mice, now in the 46th passage, produces 100 per cent. visible infection within 3 days of inoculation, but there are no clinical symptoms.

Two strains in normal mice were maintained through 9 and 24 passages respectively, but virus was only seen in a small percentage of cells in a few of the mice and both strains were eventually lost.

Plate 1.

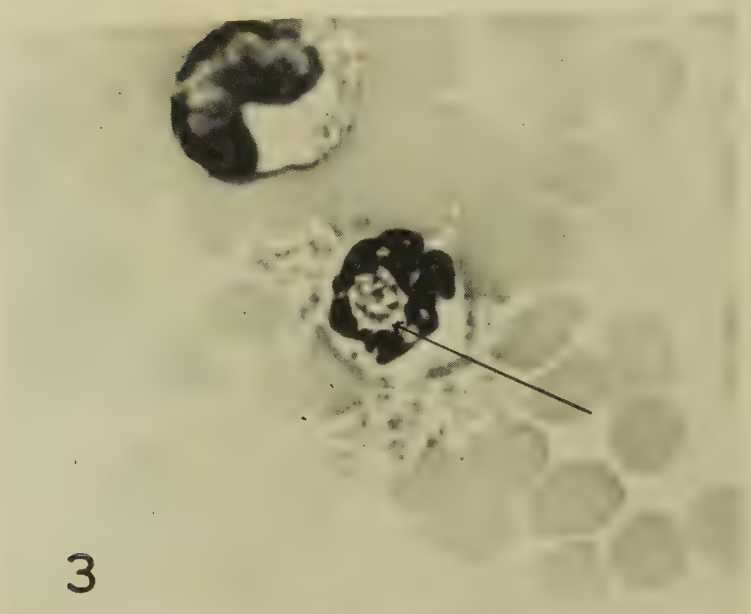




1. (A) Initial bodies in guinea-pig neutrophil.
   (B) Morula in guinea-pig neutrophil. x 1500.
2. (A) Vaoculated fragmenting forms and (B) morula in guinea-pig neutrophil. x 2400.

To face page 426

Plate 2.



3. Fragmenting form in mouse neutrophil. x 1200.

4. Fragmenting form in ruptured mouse eosinophil. The cell nucleus is surrounded by a halo of eosinophil granules. The virus cluster has been broken up showing the irregular shape of the component particles. x 2700.

Pretreatment with cortisone or nitrogen mustard failed to lower the resistance of mice to the infection.

The laboratory animal adapted strains still infect sheep.

ACKNOWLEDGMENTS

We wish to thank Mr. J. G. Brotherston who carried out spleen biopsies and inoculations while one of us (A.F.) was absent for a prolonged period on sick leave. We are also indebted to Dr. I. Zlotnik who examined brain material for the presence of inclusions resembling the Heartwater parasite and to Mr. D. G. Watson for the microphotographs and reproductions of the figures.

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THE AEROBIC BACTERIA OF THE NOSE AND TONSILS OF HEALTHY DOGS*

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INTRODUCTION

Little is known of the bacterial flora of the upper respiratory tract of healthy animals. In this paper a survey of the incidence of 20 groups of aerobic bacteria in the nose and tonsils of dogs is described.

MATERIALS AND METHODS

Swabs were taken from 100 dogs from the London area which had been brought to the Royal Veterinary College for destruction at the owner's request; care was taken to exclude dogs which were not healthy. One swab from both nostrils and one from the tonsils were cultured within an hour of collection on 5 per cent ox blood agar plates which were inspected after 24 and 48 hours at 37°C and again after a week at room temperature. Subcultures were made from each colony type observed. The morphological, cultural and biochemical characteristics of the strains were determined by conventional methods and they were assigned to groups listed in Table I. Thirty of the swabs were also examined for pneumococci by incubation for 24 hours in 5 ml. beef infusion broth of which 0·5 ml. was then injected subcutaneously into a 20 to 25 g. mouse. The heart blood of each mouse dying within 14 days was cultured on to blood agar; subcultures of suspicious colonies were examined morphologically and by the bile solubility test.

The terms 'mean percentage plate count', which gives a measure of the heaviness of each bacterium in the primary plate cultures and 'percentage incidence as dominant flora' were defined in a previous paper (Smith, 1955).

RESULTS

The primary cultures were very varied; it was usual to isolate two or three species of bacterium from the nasal cultures and four or five from the tonsillar cultures. All of the bacteria listed in Table I were found from time to time except for *Diplococcus pneumoniae* and *Bordetella bronchiseptica.*

Staphylococci

These were isolated from the nose more frequently than any other bacterium, usually in heavy culture and often forming the dominant nasal flora. They were less common in the tonsils and the low percentage plate counts suggested that their presence here was merely secondary to their presence in the nose. Pigment production was noted from subcultures on 5 per cent milk agar after 24 hours at 37°C followed by a week at room temperature. The majority (93/107) of the strains were *Staphylococcus albus* but 10 were *Staph. aureus* and

* Part of a thesis for the degree of Ph.D., University of London.

TABLE 1

OCCURRENCE OF VARIOUS BACTERIA IN THE NOSE AND TONSILS OF 100 DOGS

Bacterium	*Percentage carrier rate*		*Percentage incidence as dominant flora*		*Mean percentage plate count*	
	Nose	*Tonsils*	*Nose*	*Tonsils*	*Nose*	*Tonsils*
Staph. aureus, haemolytic	3	3	6	0	65	3
Staph. albus, haemolytic	61	38	31	4	51	13
Staph. albus, non-haemolytic	20	5	12	0	50	3
Non-haemolytic, pigmented staphylococci	4	0	1	0	26	—
Beta-haemolytic streptococci	11	31	2	12	25	32
Alpha- and non-haemolytic streptococci	24	65	14	26	55	41
Diplococcus pneumoniae	0	0	0	0	—	—
Corynebacterium spp.	3	1	2	0	44	2
Bacillus spp.	12	5	1	0	9	13
Neisseria spp.	10	20	3	1	53	28
Pasteurella septica	10	54	6	36	58	51
Haemophilus spp.	-/-	-/-	-/-	-/-	-/-	-/-
Bordetella bronchiseptica	0	0	0	0	—	—
Alcaligenes spp.	14	24	2	5	33	27
Escherichia coli and related bacteria	32	39	14	11	53	42
Proteus spp.	11	13	3	2	—	—
Leptotrichea spp.	3	10	0	0	13	15
Flavobacterium and *Achromobacter* spp.	5	21	2	2	8	24
Streptobacillus spp.	6	21	1	1	11	28
Mycobacterium spp.	-/-	-/-	-/-	-/-	-/-	-/-
Unidentified bacteria	4	12	0	0	13	15

-/- Occasionally isolated.

four resembled *Staph.aureus* except that they were non-haemolytic and coagulase-negative. Altogether 84 strains were haemolytic; beta haemolysin production was common and the alpha haemolysin was seldom seen except in combination with the beta haemolysin. About half the strains (54), including many which were beta lysinogenic, were coagulase-negative when tested by the method of Fisk (1940), but strains producing the alpha haemolysin either alone or together with beta haemolysin were invariably coagulase-positive. It is intended to report more fully on these findings in a later paper.

Streptococci

Tonsillar carriers of beta-haemolytic streptococci were commoner than nasal carriers and only three of the dogs with positive cultures yielded the organism from the nasal swab only. Thirty-five strains were examined by a modification of Lancefield's precipitin technique, using growth scraped from blood agar plates for making the extracts, with the following results: Group C (1), Group G (21), Group L (5), Group M (2); 6 strains gave no reaction with any available serum (A, B, C, G, L and M).

Alpha-haemolytic streptococci appeared to form, with *Pasteurella septica*, the basal flora of the tonsils, but non-haemolytic forms were rarely seen. No special studies were made on the strains isolated

and it is not known how closely they resemble the streptococci of the human mouth and throat. Typical, highly mucoid colonies of *Strep. salivarius* developed however from three of a series of twenty tonsil swabs with which the experiment of plating on to 5 per cent sucrose agar was tried. *Diplococcus pneumoniae* was never isolated. The mice which died after receiving injections of broth cultures yielded *Pasteurella septica* and, less frequently, beta-haemolytic streptococci, *Escherichia coli* and strains of *Proteus*.

Corynebacterium

Only three diphtheroids were found. They resembled each other closely but could not be identified with any known *Corynebacterium* species. The colonies on blood agar were 0·5 mm. diameter after 24 hours incubation and showed a milky translucence; they were not haemolytic. Urease and indole were not produced and none of the strains affected solid serum, gelatin or litmus milk. Starch was slowly hydrolysed and acid was produced from glucose and maltose but not from other carbohydrates.

Neisseria

The Gram negative cocci fell into two groups resembling the *Neisseria catarrhalis* and *N. flava* groups of the human throat. Non-chromogenic strains generally gave the reactions of a typical *N. catarrhalis*. Those producing yellowish pigment were more variable; a few appeared to be *N.flavescens* but the majority, although apparently of the *flava* group, did not fit exactly into any of the recognised subgroups.

Pasteurella septica

This bacterium was commonly isolated in heavy culture from the tonsils. Although the heterogenicity of the species is well known, dog strains seemed to possess certain special characteristics: they were often non-capsulated, saline- and acid-agglutinable and of low pathogenicity to laboratory animals; acid was produced from maltose and trehalose as well as from glucose and sucrose which are fermented by all *Past.septica* strains, but xylose and mannitol were rarely attacked. These results have already been reported (Smith, 1955, 1958).

Haemophilus

Although no special search was made, V-dependent strains were sometimes noticed on the primary plates on account of their satellitism around staphylococcus colonies.

Bordetella bronchiseptica

This species was not isolated from any of the dogs, but many cultures bearing a superficial resemblance to it were obtained. They differed however in being non-motile and in failing to grow in Koser's citrate or on desoxycholate citrate agar and to split urea; furthermore, erythrocyte agglutination never occurred when a

loopful of a thick saline suspension of a culture was mixed on a slide with a loopful of human blood. They were classified as *Alcaligenes* species because of their inability to ferment carbohydrates and their production of alkalinity in litmus milk or on litmus milk agar.

Other Gram negative Bacteria

It will be seen from the Table that a number of *Achromobacter* and *Flavobacterium* strains were recorded. A few of these produced pale yellow pigment, were non-motile, fermented no carbohydrates and had no effect on litmus milk or gelatin; these were classified as *Flavobacterium*. The majority however were non-chromogenic, non-motile rods growing freely on nutrient agar and McConkey's agar in the form of large, moist, whitish colonies; acid was produced in glucose only and the indole test was negative. A serum prepared against one strain gave strong slide agglutination with nearly all the others. Such a homogeneous group would seem to merit more detailed study with a view to accurate classification; meanwhile it may be regarded as belonging to the genus *Achromobacter*.

In a few cultures, especially those from the tonsils, small granular or rhizoid colonies were noticed; they were about 0·3 to 0·6 mm. in diameter after 24 hours and increased in size after further incubation. Smears revealed tangled masses of thick, non-branching, Gram negative filaments, often with swollen ends and usually containing abundant Gram positive granules. A few strains were examined biochemically and seemed to be highly inactive. Cultures kept at various temperatures never sporulated but died in a few weeks. It was concluded that these strains were related to or identical with the *Leptotrichea buccalis* of the human mouth (Rosebury, 1944).

The tonsils also yielded many cultures of an unusual Gram negative bacillus which does not seem to have been described previously. Most strains were pleomorphic, forming rods of about 0·5 to 1·5 μ, elongated and S-shaped forms and long, curving chains and filaments bearing densely staining, globular or pyriform bodies resembling those produced by *Streptobacillus moniliformis*. Poorly-staining, swollen forms were also common and the bacteria were usually embedded in a pale, semi-homogeneous matrix which appeared to be composed of degenerated organisms. After 24 hours incubation colonies on blood agar were 0·5 mm. diameter, non-haemolytic, greyish-translucent, umbonate and firm, enlarging later to 1·5 to 1·8 mm. In broth most strains gave a heavy, cottonwool deposit and the supernatant remained clear; others formed a granular deposit. Slight acidity was produced from glucose, maltose and sucrose, but not from other carbohydrates and the indole test was negative. Injection of broth cultures into the peritoneum and hind foot pad of mice produced no effect and a serum prepared against one strain did not agglutinate a suspension of *Streptobacillus moniliformis*. The ready growth under dry, aerobic conditions, absence of L-type colonies, non-pathogenicity to mice and failure to give

cross-agglutination indicated that the organism was not *Streptobacillus moniliformis*, but it seemed to fit well into the genus *Streptobacillus* and during the course of the survey it was found convenient to refer to it as *Streptobacillus canis*.

Enteric Bacteria

Coliforms appeared surprisingly often in both nasal and tonsillar cultures. Thirty of these were examined biochemically with the following results: *Escherichia coli* Faecal Type I (24), Intermediate Type I (3), Intermediate Type II (1), *Klebsiella aerogenes* Type I (1), Paracolon (1). A high proportion (11/30) of the strains were haemolytic on ox blood agar. Thirteen of the 14 Proteus strains were *Pr. mirabilis* and the remaining one *Pr. vulgaris*.

DISCUSSION

Because of the marked individual variation found, it is not possible to describe a basal flora of the upper respiratory mucosa present in all dogs under a wide variety of conditions. One can however recognise a certain range of flora whose presence might be predicted in about half of any series of household dogs examined. This characteristic flora in the case of the nose is composed of beta haemolysinogenic *Staph.albus* while the characteristic tonsillar flora comprises alpha- and beta- haemolytic streptococci together with a certain type of *Past.septica* which is apparently confined to dogs. These may occur alone or mixed with coliform organisms, Gram negative cocci and many other bacteria including a great variety of Gram negative rods of low fermentative ability.

Despite the fact that most household dogs are allowed little association with others of their own species the results of the survey suggest that the greater part of their respiratory flora must be acquired from other dogs. For example, the group G beta-haemolytic streptococci may be assumed to have been derived in this way since they are rarely isolated from any other host species (Hare and Fry, 1938); the particular type of *Past.septica* found in dogs' tonsils is not known to occur elsewhere except in dogbite wounds (Smith, 1958, 1960); the common nasal staphylococcus of dogs is a beta haemolysinogenic *Staph.albus* which occurs characteristically in animals including dogs (Minett, 1936; Smith, 1947), but is rare in the human species which is the dog's most frequent contact. Moreover, the particular types of *Neisseria* found during the survey, the organism tentatively named *Streptobacillus canis* and the group of Gram negative rods classed as *Achromobacter* do not seem to have been described before and may well be peculiar to the dog. It seems likely that a respiratory flora of a more or less adult type is acquired early in life by contact with other dogs, perhaps from the mother, for the two youngest dogs examined, both eight weeks old, yielded cultures indistinguishable from those given by adult dogs.

Although it is unlikely that much interchange of respiratory flora

takes place between the dog and its human owner a few alpha-lysinogenic *Staph.aureus* strains were isolated during the survey and may have come from human sources; the occasional Group A streptococcus obtained from dogs may be of similar origin (Hare and Fry, 1938; McFadden and Boon, 1949). Transmission in the other direction, from dog to human, has not been recorded.

The commonest pyogenic bacterium in dogs is known to be the beta haemolysinogenic variety of *Staph.albus* which is found in a large proportion of cases of furunculosis and in other suppurative conditions. The high nasal carrier rates obtained for this organism suggest that most of such cases are endogenous infections derived from the animal's own nasal flora. The same probably applies to pyogenic conditions due to beta-haemolytic streptococci. Disease due to *Past.septica* is rare in adult dogs, but the organism may act as a mild secondary pathogen in respiratory infections including pneumonia. Needless to say, the isolation of any of these bacteria from the nose or tonsils of diseased dogs would carry little or no pathological significance. *Bordetella bronchiseptica* however is occasionally found in chronic and intractable laryngotracheitis and in view of its absence from the dogs in the survey may be regarded as the causal organism in such cases.

CONCLUSIONS

Cultures from the nose and tonsils of 100 household dogs were examined for 20 groups of aerobic bacteria. The basal flora of the nose comprised beta haemolysinogenic, coagulase-variable strains of *Staph.albus*, and that of the tonsils *Past.septica* and alpha-haemolytic streptococci of undetermined types. *Bordatella bronchiseptica* was not found, but *Alcaligenes* strains bearing a superficial resemblance to it were common. *Neisseria* strains resembling, but not identical with *N. flava* and *N.catarrhalis* were isolated. Some of the dogs also carried a hitherto unrecorded *Streptobacillus* species and a Gram negative rod of low fermentative ability tentatively classed as *Achromobacter*.

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PAPAIN-TREATED RED CELLS IN THE DETECTION OF PIG RED CELL ISO-ANTIBODIES

By

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INTRODUCTION

Enzymes such as trypsin, papain and ficin have been found to alter human red blood cells, so that they are agglutinated by incomplete antisera which otherwise do not agglutinate saline suspensions of normal red cells. Most pig iso-antibodies are of the 'incomplete' type and the method used so far (Saison, Goodwin and Coombs, 1955) has been the indirect sensitization test (I.S.T.). The washing needed in this test is laborious, especially when working with large numbers of titrations. The action of papain and ficin on pig red blood cells was studied in the hope of finding a test as reliable as the I.S.T. but less tedious for routine use.

Certain antigens on human red blood cells are known to be destroyed by the enzyme trypsin (Morton and Pickles, 1951; Unger and Katz, 1951, 1952) and, according to Hubinont (1951) and Weiner and Katz (1951), enzyme-treated cells absorb antibody more completely than untreated cells. In the course of this work these properties of enzyme-treated cells discovered by workers on human cells were also studied.

MATERIALS AND METHODS

Sera. The iso-sera used were those described by Joysey, Goodwin and Coombs (1959).

Red blood cells. The blood was obtained from a panel of farm pigs at the School of Veterinary Medicine, Cambridge; four volumes of blood were taken into one volume of 3·4 per cent (w/v) tri-sodium citrate ($Na_3\ C_6\ H_5\ O_7\ 2.\ H_2O$). Citrated blood was kept at 4°C and used not later than six days after bleeding. Before use the cells were washed three times and suspended in 0·85 per cent saline maintained at pH 7·2 with Sørenson's buffer (10 cc. M/15 buffer to each litre of saline). Between each wash and before the first wash the cells were incubated in a water bath at 37°C for five minutes.

Papain and papain test. The papain (B.D.H.), buffer and activator (cysteine hydrochloride) were prepared according to the method of Stratton (1953). Fresh preparations of buffer and of activator were made each week and stored at 4°C; the papain was stored in 5 ml amounts at -20°C for a number of months.

Red blood cells were washed three times. To a 20 per cent suspension of red cells was added an equal volume of a freshly prepared mixture of one volume of 0·25 per cent papain, one volume of buffer and two volumes of 0·2 per cent activator. The mixture was incubated in a water bath at 37°C. After twenty minutes the cells were washed once and saline was

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added to give a 2 per cent suspension. One drop of the cell suspension was added to one drop of serum dilution in a small tube (5 x 50 mm) and incubated for one and a half hours at 37°C. The sedimented cells were then transferred to a slide with a Pasteur pipette and examined with a low power microscope.

Ficin and ficin test. The method used for preparing the ficin (Light's) was that of Race and Sanger (1958). Nine volumes of a 2 per cent suspension of red cells, washed three times, were treated with one volume of a 0·25 per cent solution of ficin for fifteen minutes in a water bath at 37°C. The treated cells were washed twice and resuspended with saline to give a 2 per cent suspension. One drop of the treated cells was added to one drop of serum dilution in a small tube (5 x 50 mm) and incubated at 37°C for one and a half hours. The test was read by the same method as the papain test.

Where cells for absorption were treated with ficin, one volume of a one per cent solution of ficin was added to nine volumes of a 20 per cent suspension of washed cells and the mixture heated in a 37°C water bath for thirty minutes. The cells were then washed twice and finally packed. The absorption of the pig iso-antisera with the ficin-treated cells was carried out as described for papain-treated cells.

Indirect sensitization test. The indirect sensitization test was performed as described by Saison *et al.* (1955) except that all incubations were at 37°C.

Absorption of iso-antisera with papain-treated cells. The cells were washed three times. They were then packed by centrifugation at 3,000 r.p.m. for fifteen minutes. Half of the packed cells were removed for treatment with papain, the other half were left as untreated control cells. To a 20 per cent suspension of the washed cells was added an equal volume of the same mixture as previously described, except that it was made with one per cent papain instead of 0·25 per cent. This was then placed in a water bath at 37°C for three quarters of an hour. Subsequently the cells were washed twice and again packed. An equal volume of iso-antiserum was added to both treated and untreated cells except where otherwise indicated. After incubation at room temperature for ten minutes the cells were deposited by centrifugation and the supernatant sera removed. Further absorptions of the sera were at 37°C for the same length of time.

RESULTS

Papain Test as a Routine Test

Papain-treated cells were used in parallel with the indirect sensitization test. The results are shown in Table I. It was found that the titre, using enzyme-treated cells, was the same as that obtained with the I.S.T. or one serial dilution less. With the papain test, however, three sera, anti-6, anti-8 and anti-9 gave little or no reaction with 6 positive, 8 positive or 9 positive cells respectively.

Destruction of Antigens 6, 8 *and* 9

The weak reaction or absence of reaction of papain-treated cells with anti-6, anti-8 and anti-9 was thought to be due to the destruction of these antigens by the enzyme. These sera were therefore absorbed with untreated positive, papain-treated positive, untreated negative

TABLE 1

COMPARISON OF THE PAPAIN TEST AND THE I.S.T. USING THE ROUTINE REAGENTS

Reagent	*Titre of serum obtained with:*	
	I.S.T.	*Papain*
Anti-A ..	1024	512
Anti-1 ..	32	32
Anti-2 ..	32	32
Anti-3 ..	512	256
Anti-5 ..	128	64
Anti-6 ..	256	4
Anti-8 ..	128	0
Anti-9 ..	256	0

With both tests no agglutination was obtained with cells lacking the antigen concerned.

TABLE 2

ABSORPTION OF THREE REAGENTS WITH PAPAIN-TREATED AND UNTREATED CELLS TO SHOW THE DESTRUCTION OF THE ANTIGENS CONCERNED

Reagent	*Reagent absorbed by:*	*Titre of antibody as shown by I.S.T.*
Anti-6	Unabsorbed	256
	Papain-treated 6—	64
	Untreated 6—	64
	Papain-treated 6+	64
	Untreated 6+	0
Anti-8	Unabsorbed	128
	Papain-treated 8—	64
	Untreated 8—	64
	Papain-treated 8+	64
	Untreated 8+	0
Anti-9	Unabsorbed	256
	Papain-treated 9—	64
	Untreated 9—	128
	Papain-treated 9+	64
	Untreated 9+	0

TABLE 3

ABSORPTION OF PIG ANTI-A WITH PAPAIN-TREATED AND UNTREATED CELLS TO SHOW THE DIFFERENCE IN THE TWO ABSORPTIONS

Number of absorptions	*Titre of pig anti-A by I.S.T. after absorption with:*	
	Papain-treated A cells	*Untreated A cells*
Unabsorbed	1024	
1	64	512
2	8	64
3	2	4
4	1	4
5	0	2
6	0	1

TABLE 4

ABSORPTION OF PIG SERA WITH FICIN-TREATED AND UNTREATED CELLS TO SHOW THE REMOVAL OF THE 'PANAGGLUTININ' TO FICIN

Serum	*Serum absorbed by:*	*Titre of panagglutinin as shown by I.S.T. using absorbing cell as test cell*
Pig 132	Unabsorbed	16
	Ficin-treated 132	0
	Untreated 132	4
Pig H.T.	Unabsorbed	128
	Ficin-treated H.T.	2
	Untreated H.T.	32
Reagent Anti-A	Unabsorbed	32
	Ficin-treated O cell	0
	Untreated O cell	8

and papain-treated negative cells. The resulting sera were tested for the antibody in question by the I.S.T. In all cases, as shown in Table 2, the titre of the antibody was only slightly reduced by absorption with positive cells after treatment with the papain, whereas the untreated positive cells completely removed the antibody after five absorptions.

Absorption of Pig Anti-A with Papain-treated and Untreated Group A Pig Cells

Wheeler, Luhby and Scholl (1950) and Hubinont (1951) claimed that trypsin-treated human red cells absorbed more anti-D than did normal cells. Aliquots of pig anti-A were absorbed with papain-treated and untreated pig A cells. The volume of serum used was twice that of the packed cells. Table 3 shows that the papain-treated cells completely removed anti-A after five absorptions whereas the sera absorbed with the untreated cells still possessed detectable antibody after six comparable absorptions.

Treatment of Pig Cells with Ficin

All pig sera panagglutinated ficin-treated red cells. Cells treated with ficin were agglutinated by the routine antisera and by the pig's own serum. Table 4 shows that after five absorptions with ficin-treated cells the panagglutinin against ficin-treated red cells was removed (except in one case where a titre of 1:2 was still obtained) whereas untreated red cells did not remove it.

Production of a Pure Anti-9 Reagent

The anti-9 serum produced by Joysey *et al.* (1959) also contained anti-4 and anti-5. The anti-4 and anti-5 could not be removed because no 9-negative, 4- and 5- positive pig was available. It was hoped that a 9-negative, 4- and 5-positive cell could be produced artificially by treating 9-, 4- and 5- positive cells with papain, and by destroying antigen 9, the anti-4 and anti-5 might be absorbed, leaving only anti-9.

In a preliminary test it was found that treatment with papain for one hour was needed before antigen 9 was no longer detectable by the indirect sensitization test. The anti-9 reagent was therefore absorbed with cells that had been treated for one hour. When the resulting serum was tested by the I.S.T. it was found that, as hoped, anti-9 was present at a titre only one tube lower than that in the unabsorbed serum, whereas the anti-4 and -5 had been considerably reduced. However, a further iso-antibody, previously unsuspected, was discovered at a titre of 1:16. This made it impossible to tell whether the anti-4 and -5 had been completely removed. Had the titre of anti-9 been higher than 1:256 it might have been practicable to use this reagent at a dilution greater than 1:16. Nevertheless, the method of destroying an antigen, in order to obtain a cell with the required antigenic structure, may be of value in such a problem as that described.

CONCLUSIONS

The papain test was found to compare favourably with the indirect sensitization test as a routine method for measuring certain iso-antigens on pig red blood cells. Its use is limited however, as certain antigens are destroyed by the enzyme. When possible the

papain test is preferable because less washing is needed. Further, the microscopic reading of the I.S.T. when using pig cells tends to be rather difficult. A somewhat granular appearance with two or three cells clumped together in an otherwise negative field is often found, except when using the cells of piglets. The papain test, however, gives a clearer result with a sharper end point.

It was shown that antigens 6, 8 and 9 are destroyed by the enzyme. Cells having these antigens when treated with papain were no longer capable of absorbing the corresponding antibody. This phenomenon was used to try to obtain a pure sample of our reagent anti-9, but this was not successful as an unknown iso-antibody was encountered which masked the result. However, the method might succeed with another serum in which the desired antibody was of sufficiently high titre.

Papain-treated pig A cells were shown to absorb pig anti-A more rapidly and more completely than untreated cells. Since many absorptions are often needed to remove pig iso-antibodies from a serum, absorption of pig sera with papain-treated cells would be of advantage.

Ficin has also been tested, but no conclusions as to the routine use of this enzyme can be drawn, as what appeared to be a panagglutinin to ficin-treated cells was encountered. This panagglutinin could be absorbed by ficin-treated cells but not by untreated cells.

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THE HISTOPATHY OF THE BRAIN OF GOATS AFFECTED WITH SCRAPIE

By

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INTRODUCTION

The experimental transmission of scrapie from sheep to goats has created a considerable amount of interest in the pathology of scrapie in the goat. Thus Pattison, Gordon and Millson (1959) found vacuolated and degenerated neurones in the medulla of goats affected with experimental scrapie and Hadlow (1959) observed degeneration of neurones and astrocytic proliferation, not only in the medulla but along all the levels of the neuraxis.

In the present study, a detailed description of the lesions in the various parts of the brain of the goat affected with scrapie is given and an attempt is made to correlate the incidence of the lesions with the clinical forms of the disease.

MATERIALS AND METHODS

Brains from 50 goats have been examined in the present study. Of these, forty-two brains were obtained from goats suffering from an obvious clinical form of scrapie induced by intracerebral inoculation of 1 ml. of brain emulsion from scrapie sheep or goats (Mackay, Smith and Stamp, 1960). Seven brains were obtained from uninoculated apparently healthy goats which served as normal controls. One brain included in this study originated from a natural case of scrapie in an uninoculated goat (Mackay and Smith, 1961).

All the goats were destroyed by exsanguination within 1 to 5 weeks after the onset of symptoms. The brains were removed immediately after death, fixed for about 14 days in 15 per cent formol-saline and then post-fixed in corrosive sublimate for 2 days. Serial paraffin sections 7 μ in thickness were stained with haematoxylin and eosin. The Nissl substance was examined in paraffin sections stained with eosin and methylene blue and the method of Holmes was used for the demonstration of neurofibrils. Myelin was stained as a rule in formol-fixed frozen section according to the methods of Spielmeyer or of Smith and Quigley. Sudan IV was used for the demonstation of fat, and Cajal's gold chloride and sublimate technique gave excellent results for the demonstration of astrocytes.

The following parts of the brain were examined: medulla-cord junction, medulla, pons, mesencephalon, diencephalon, corpus striatum, gyrus subcallosus, hippocampus, cerebral cortex and cerebellum.

RESULTS

Lesions in Brain

Lesions of varying intensity were found in all the 42 experimental scrapie goats and in the one natural case. Significant lesions were absent in the seven control goats. With the exception of the cerebral

cortex all parts of the brain showed pathological change, but the distribution of the lesions and their severity varied from animal to animal. Certain parts of the brain were always affected, but in other parts the location and incidence of lesions differed from brain to brain.

The principal microscopic lesion in all the scrapie goats was focal or diffuse degeneration of the grey matter of the brain. The degenerative process affected primarily the neurones, but in many instances the intercellular ground substance was also diseased. The extent of the neuronal damage varied: in some cases only a few cells were degenerated but in many other cases large areas of the brain were found almost entirely deprived of healthy or undamaged neurones. In areas of large-scale neuronal damage astrocytic proliferation was often present, but in many other centres there was no glial reaction (Figs. 1 and 2). Oligodendroglial proliferation and increased perineural satellitosis was a regular feature in parts of the cerebral cortex, especially in the hippocampal gyrus. Here, very often mixed infiltrations of oligo and microglial cells were clustered around the neurones (Fig. 3). Myelin changes and perivascular infiltrations were not a regular feature in scrapie goats, but in a few brains perivascular accumulation of lymphocytes and plasma cells, both within the brain tissue and in the chorioid plexus, were very great.

Various forms of neurone degeneration such as chromatolysis, pyknosis, sclerosis, necrosis, simple and multiple vacuolation were always present, but apart from these forms of degeneration two other types were noticed (Figs. 4a and b and 5a). The first type was a hyaline eosinophilic degeneration of the cytoplasm which often affected the Purkinje cells of the cerebellum and the neurones of the dentate nucleus. The nucleus in such cells usually remained in the centre of the cytoplasm and was either enlarged with all details of internal structure obliterated, or was undergoing karyorrhexis or pyknosis (Figs. 5b and 6a). The second type was found only in neurones of the rostral parts of the brain in the gyrus subcallosus and consisted of a granulo-vacuolar degeneration of the cytoplasm, the small vacuoles of which contained small eosinophilic granules (Fig. 6b). Vacuolated neurones were generally present in all the affected areas of the grey matter. Multiple forms of vacuolation often caused extreme enlargement of the cytoplasm and such vacuoles contained either eosinophilic masses of necrotic material or regular round bodies (Fig. 5a).

The intercellular ground substance was often vacuolated and this was observed both in areas where neuronal degeneration was widespread or absent. In the majority of brains only focal areas of intercellular vacuolation were present, but in animals which developed the disease after an unusually long incubation period the intercellular vacuolation assumed enormous proportions (Figs. 10, 11 and 12).

Medulla-cord junction. The medulla-cord junction was affected in a proportion of brains only, the most severe lesions being found in animals with long incubation periods. Necrosis of neurones and single large vacuoles were the most common neuronal lesion. The most regularly affected areas were the pigmented nucleus of the medulla oblongata, accessory nucleus, reticular formation and the spinal trigeminal nucleus.

Medulla. In the medulla oblongata neuronal lesions were found in almost all the goats, but except in animals with long incubation periods were neither widespread nor very severe. The areas most characteristically affected by vacuolation and degeneration were the nuclei of the midline and raphé, the formatio reticularis alba, the reticular formation, the arcuate cells and very frequently the inferior olivary nucleus (Fig. 7). Intercellular vacuolation was only rarely present, but astroglial proliferation was seen in some brains.

Pons. The severity of the pontine lesions resembled that of the medulla where the nuclei of the spinal and sensory trigeminal, the vestibular and the reticular formation were very often affected. In animals with widespread neuronal damage the intercellular ground substance was also vacuolated.

Mesencephalon. The mesencephalon was invariably affected in all scrapied goats, but the lesions were not equally distributed in the various regions. Thus the rostral part of the mesencephalon contained fewer lesions than the middle and caudal parts. Neuronal lesions were often present in the substantia nigra and occasionally in the superior colliculus. On both sides of the rostral part of the midbrain the intercellular ground substance of the medial geniculate bodies was often vacuolated, especially in goats with the very long incubation period. In the middle part of the mesencephalon the red nucleus showed the most marked degeneration where the most common changes were chromatolysis, necrosis and vacuolation. The vacuoles were unusually large and contained multiple cavities with intravacuolar bodies (Figs. 4a and b, 5a and 8). This exceptional striking picture of vacuolation of the red nucleus was present in all the animals with the exception of goats that showed the rubbing syndrome, where only chromatolysis was present. In the naturally affected goat the vacuoles were small and formed clusters within the neurones. The other nuclei of the middle of the midbrain were also affected, especially the motor-tegmento-peduncular nucleus, the pigmented and the substantia nigra. In the caudal part of the mesencephalon, severe and constant lesions were invariably present within the nuclei of the tegmentum, the dorsal nucleus of the raphé, the superior central nucleus, the ventral and dorsal tegmental nuclei and the reticulo-tegmental nucleus. The neurones of these nuclei were always vacuolated, but other degenerative forms were also present. Apart from the tegmentum the nucleus of the inferior colliculus was affected in a large proportion of brains.

Diencephalon. Both vacuolation and degeneration of neurones

were equally present in the thalamic nuclei and in the hypothalamus, especially in the mammillary body, lateral mammillary nucleus and in the paraventricular nucleus. In addition to cellular changes intercellular vacuolation assumed enormous proportions here. In fact in some of the goats that had a very long incubation period, large areas of softening were present. The microscopical picture of these softened centres resembled a network of fibres and strands with only few neurones remaining. In some cases, however, in spite of the extremely severe vacuolation the neurones remained intact (Figs. 11 and 12). In some of these severely vacuolated areas of the thalamus the absence of any glial reaction was very striking. In other brains often less severely affected by vacuolation astroglial proliferation was a regular feature.

Telencephalon. In the corpus striatum lesions were not often present except in goats with very long incubation periods where severe vacuolation of the intercellular ground substance affected the lenticular nucleus. In the rostral parts of the base of the brain below the rostrum of the corpus callosum, degeneration of neurones with widespread vacuolation was of constant occurrence in all the scrapie goats. In the gyrus subcallosus vacuolation was very often exceptionally severe; degenerative changes were also seen in the area parolfactoria (Brocae) (Fig. 9).

Cerebellum. Degeneration of neurones in the various nuclei and in the Purkinje cells was common in the majority of goats. All the cerebellar nuclei were equally affected, but vacuolation was most common in the dentate nucleus. Chromatolysis, pyknosis, peripheral vacuolation, single and multiple intracytoplasmic vacuoles, eosinophilic hyaline degeneration, necrosis and atrophy were frequently present in the Purkinje cells. In some cases the granule cells were also undergoing degeneration. The glial cells, especially those next to the Purkinje cells, were often proliferating and formed a dense network (Fig. 1). The intercellular ground substance was only seldom affected in the cerebellum.

Clinico-Pathological Correlation

According to their clinical signs all the goats affected with scrapie included in the present study have been grouped into those showing the nervous syndrome and those of the scratching syndrome (Pattison and Millson, 1961). Amongst the animals that were showing only nervous symptoms some goats were exhibiting hyperexcitability and inco-ordination while other goats, especially those that developed the disease after an unusually long incubation period ranging from 29 to 40 months (Mackay *et al.* 1960), were somnolent and very severely incoordinated. Animals that were showing the scratching syndrome were divided into two sub-groups those that were affected by severe rubbing and incoordination from the onset of the disease, and those that were rubbing only at the time of death.

TABLE 1

LESIONS IN THE BRAIN OF GOATS AFFECTED WITH EXPERIMENTAL SCRAPIE

Clinical syndrome	Serial no.	Period from inoculation till destruction (in months)	Medulla				Pons				Mesencephalon							
			Neuron		Interstit.		Neuron.		Instertit.		Neuron.						Insterstit.	
											Tegment. n.		Red n.		Other n.			
			Vac.	Other degen.	Vac. inter-cell	Astro-glial	Vac.	Other degen.	Vac. inter-cell	Astro-glial	Vac.	Other degen.	Vac.	Other degen.	Vac.	Other degen.	Vac. inter-cell	Astro-glial
1. Inco-ordination	333	10	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
	360	11	+−	+−	−	−	+−	+−	−	−	+	+	+	+	−	−	−	−
	479	10	$+_2$	$+_2$	+−	+−	$+_3$	$+_3$	−	−	$+_2$	$+_2$	+	+	+	$+_2$	+	+−
	482	15	$+_2$	$+_2$	−	−	$+_3$	$+_3$	−	−	$+_3$	$+_3$	$+_3$	$+_3$	+−	+	+−	−
	512	10	+	+	−	+−	+	+	−	−	$+_2$	+	$+_2$	$+_2$	+	+	$+_2$	−
2. Severe inco-ordination and somnolence	412	30	$+_2$	+	−	+−	+	+	$+_2$	−	$+_2$	$+_3$	$+_3$	$+_3$	+	+	+−	+
	448	35	+−	$+_2$	+−	+−	+	+	+−	$+_2$	+	$+_2$	$+_2$	$+_3$	+	+	$+_3$	+
	468	37	$+_5$	$+_3$	−	$+_2$	$+_4$	$+_4$	+	$+_2$	$+_2$	$+_2$	$+_2$	$+_3$	+−	+	+	$+_3$
	484	42	$+_3$	$+_3$	−	+−	+	+	−	+−	$+_2$	+	$+_3$	$+_3$	$+_2$	+	$+_2$	+
	517	43	$+_3$	$+_3$	$+_2$	+−	+	+	$+_3$	+−	$+_3$	$+_2$	$+_3$	$+_3$	−	+	$+_3$	+
3. Rubbing and inco-ordination	417	9	$+_2$	$+_2$	−	+	+	$+_2$	−	−	$+_3$	+	$+_3$	+	−	+−	−	+−
	418	9	$+_3$	$+_3$	−	+	+−	+−	−	−	$+_2$	+	+	+−	+−	+−	−	+
	530	11	+	+	−	−	+	+	−	−	$+_2$	+	$+_3$	$+_3$	$+_3$	$+_3$	−	+−
	535	12	+	+	−	−	+	+	−	−	+	+	+	+	$+_2$	$+_2$	+	−
	539	12	+	+−	−	+−	$+_2$	$+_2$	−	−	$+_2$	+	$+_2$	$+_2$	−	−	$+_2$	−
4. Rubbing only	487	9	+	+−	−	−	+	+	−	−	+	$+_2$	+−	+	+	+	−	−
	489	$8\frac{1}{2}$	+	+	−	+−	+−	+−	−	−	+	+	−	+−	−	−	−	+−
	504	10	+	+	−	−	$+_2$	$+_2$	−	−	$+_3$	$+_2$	−	+	−	−	−	−
	510	11	+	+	−	−	+	$+_2$	−	−	$+_3$	+	−	+−	+	+	−	−
	515	10	−	−	−	−	+	+	−	−	+	+	−	+	+−	+−	−	−

Clinical syndrome	Serial no.	Period from inoculation till destruction (in months)	Diencephalon				Telencephalon								Cerebellum			
			Neuron.		Insterstit.		C. striatum				Gyrus subcallosus				Neuron.		Interstit.	
							Neuron.		Interstit.		Neuron.		Interstit.					
			Vac.	Other degen.	Vac. inter-cell	Astro-glial	Vac.	Other degen.	Vac. inter-cell	Astro-glial	Vac.	Other degen.	Vac. inter-cell	Astro-glial	Vac.	Other degen.	Vac. inter-cell	Astro-glial
1. Inco-ordination	333	10	+-	+-	+-	−	−	−	−	−	+-	+-	−	−	−	+-	−	−
	360	11	+	+	+-	−	−	−	−	−	+-	+-	−	+-	−	+-	−	−
	479	10	+	+	+	+-	+-	+-	−	−	+	+	+	+	+2	+3	+	+3
	482	15	+	+	+	−	−	−	−	−	+	+	+	−	−	+	−	−
	512	10	+	+2	+	+-	−	+	+	−	+3	+3	+2	+	+-	+2	−	−
2. Severe inco-ordination and somnolence	412	30	+3	+3	+5	+-	−	+	+3	−	+3	+3	+2	+	+2	+2	+	+
	448	35	−	+2	+5	+	−	+	+2	+	+	+	+3	+	+	+	+	+
	468	37	+-	+	+2	+2	−	−	+	+	+	+	+	+	+-	+2	+3	+3
	484	42	+2	+3	+3	+-	+	+	+	−	+	+	+	+	+	+	−	+-
	517	48	+2	+2	+4	+2	−	+	+3	−	+3	+3	+4	+	−	+-	−	+-
3. Rubbing and inco-ordination	417	9	+2	+	+	+	−	−	−	−	+	+	+	+	+2	+2	−	−
	418	9	+	+	+	+	+-	+-	+	−	+	+	+	+2	−	+2	−	−
	530	11	+2	+2	+3	−	−	−	+	−	+3	+3	+2	+	+3	+3	−	+-
	535	12	+	+	+3	−	−	+	+3	−	+2	+2	+	−	+-	+-	−	−
	539	12	+-	+2	+2	−	−	−	−	−	+3	+3	+2	−	+-	+	−	−
4. Rubbing only	487	9	−	−	−	−	−	−	−	−	+-	+-	−	−	−	+-	−	−
	489	8	+	+2	+	+-	−	−	+-	−	+-	+-	−	+-	−	+-	+-	−
	504	10	+	+	+-	−	−	−	−	−	+-	+	−	−	−	+-	−	−
	510	11	+4	+3	+-	−	−	−	−	−	+	+	−	−	−	+-	−	+
	515	10	+2	+	+2	+-	−	−	−	−	+3	+3	+	−	−	+-	−	−

+- — +5 various degrees—from small focal lesions—to very large areas severely affected.

Five animals of each of the four sub-groups were chosen at random and the distribution and severity of the lesions in six consecutive serial sections of various parts of the brain were summarised in Table 1. It is obvious that, irrespective of the syndrome of the disease, the most constant neuronal lesions were present in the tegmental nuclei of the mesencephalon and to a lesser degree in the rostral part of the brain in the gyrus subcallosus and area parolfactoria. The red nucleus of the midbrain was affected by degenerative changes in all the goats, but vacuolation was present in animals that showed some degree of incoordination of gait at the time of death and was absent from animals that were rubbing only. As a whole, vacuolation was widespread in many parts of the brain including the diencephalon, but was absent from the corpus striatum. In the cerebellum some Purkinje cells were always degenerated, but vacuolation of Purkinje cells and of neurones in the cerebellar nuclei was absent from animals that showed rubbing only. Interstitial changes were very common in the scrapie goats, but while astrocytic proliferation could be found anywhere along the neuraxis, intercellular vacuolation was most prominent in the rostral parts of the brain, especially in the diencephalon. The most severe vacuolation of the intercellular ground substance that caused focal rarefications and softenings was present as a rule in all the goats with very long incubation periods.

DISCUSSION

The histopathology of the brain in goats is similar in many respects to that of sheep affected with scrapie (Zlotnik, 1958a & b, Zlotnik, 1960; Zlotnik and Katiyar, 1961). The incidence of the lesions in the brain of goats and their distribution in the various parts is much more regular than in the sheep. It appears therefore that the goats represent a much better subject than the sheep for the study of the pathology of the brain in scrapie.

Hadlow (1959) stressed the importance of the thalamic lesions in goats affected with scrapie and suggested that these changes may form a morphological basis for the understanding of the visual impairment evident in many goats, particularly in the advanced stages of the disease. This may be so and the importance of this observation cannot be questioned. At the same time the damage to the thalamus cannot explain the whole pathology of scrapie in view of the fact that thalamic lesions in the early stages of the disease are very scanty and that blindness and impaired vision are not a regular feature in scrapie goats, at least in the writer's experience.

The histological findings of the present study suggest that, irrespective of the stage or form of the disease, the most constant and most regular lesions occur in the tegmentum of the mesencephalon. The majority of lesions in other parts of the brain follow the natural anatomical connections of the tegmental nuclei and the red nucleus along the raphé of the pons and medulla, to the cerebellum

and rostrally to the thalamus. Thus lesions in the red nucleus and cerebellum may explain to a certain extent incoordination and disturbances in muscular tonus, while damage to hypothalamic nuclei will cause somnolence. It is worth noting that in those goats in the group showing the scratching syndrome which were destroyed soon after the onset of symptoms and before they could have developed any evidence of incoordination, lesions in the red nucleus were very limited and vacuolation was almost absent.

In addition to lesions in the tegmentum and the rubro-cerebellar system many other centres were affected such as the thalamus and superior colliculi which control visual and other activities, and the inferior colliculi and the vestibular nuclei which coordinate auditory impulses and body balance. Damage to the spinal and sensory trigeminal nuclei in the pons may explain disturbances in tactile sensibility. Finally, changes in some of the olfactory connections (area parolfactoria and hippocampus) may cause disturbances which are not yet properly assessed.

The actual lesion in scrapie of goats like that of the sheep is a progressive degeneration of the grey matter where, apart from the usual forms of neuronal degeneration, vacuolation of the cytoplasm assumes such an importance that one is compelled to consider vacuolation, especially of the multiple foamy variety, as characteristic of the disease. In some quickly progressive clinical cases, apart from vacuolation and degeneration, necrosis of neurones is very obvious. Vacuolation of the intercellular ground substance is a definite feature of scrapie in the goat, but is not commonly seen in the sheep. It appears that the degree of this form of intercellular degeneration or oedema is in direct relationship to the length of the incubation period. In animals with an unusually long incubation period the intercellular vacuolation is very severe and causes softening of whole centres of the midbrain and thalamus.

CONCLUSIONS

Progressive degeneration of the grey matter of the brain forms the principal pathological lesion in scrapie of goats.

Vacuolation of neurones and other forms of degeneration including necrosis are commonly present in most parts of the brain. In the cerebral cortex the only part affected by neuronal degeneration is the gyrus subcallosus.

The most constantly affected part of the brain of goats is the tegmentum and its connections.

Intercellular vacuolation of the brain is often present and becomes very severe in cases where the incubation period was unusually prolonged.

Some of the lesions in the brain furnish a morphological basis for the explanation of the clinical symptoms of scrapie.

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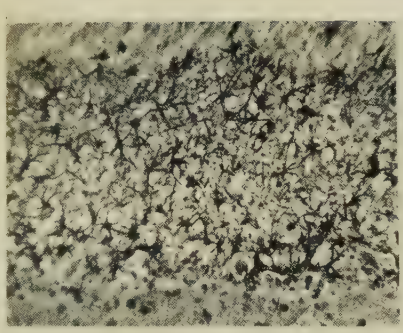
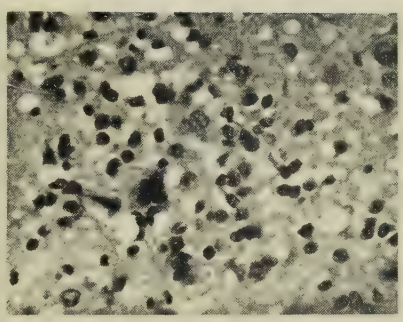
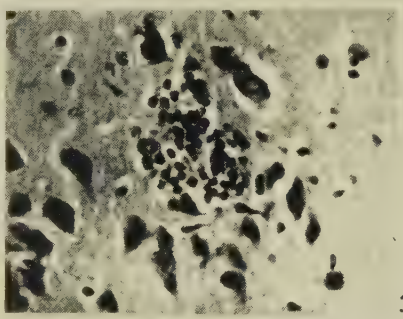
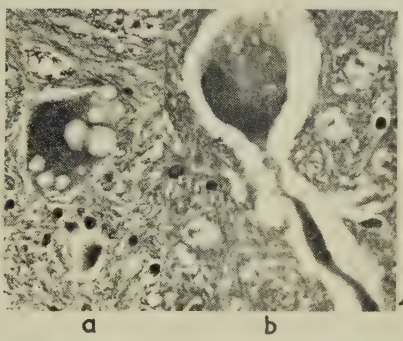
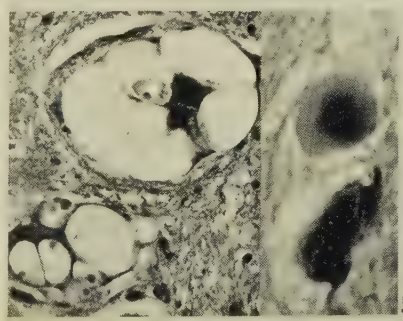










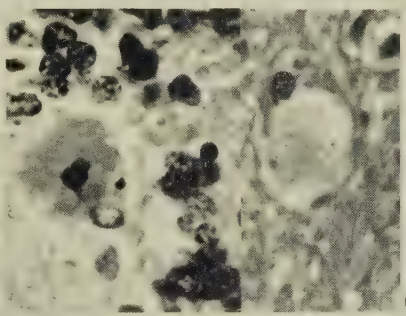
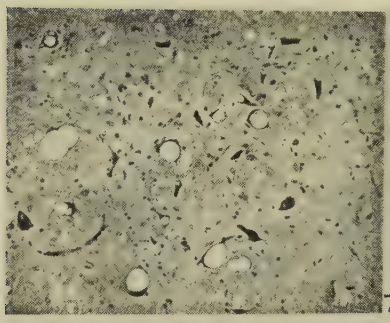
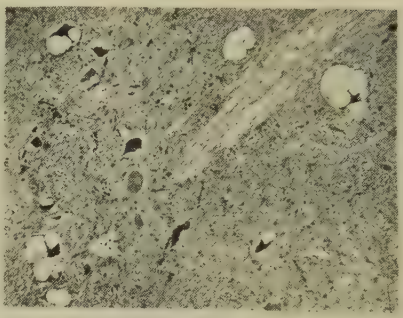
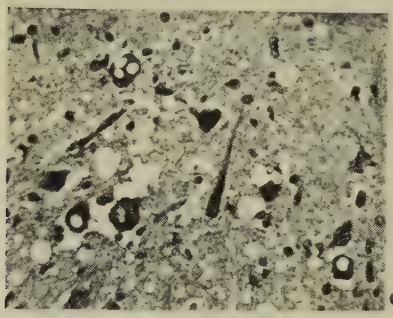


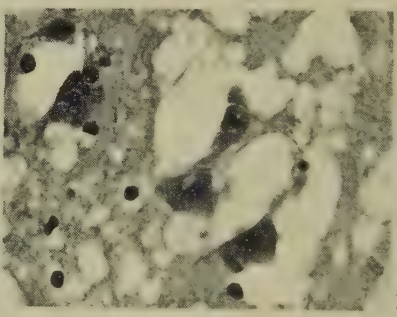
Fig. 1. Proliferation of astrocytes in the cerebellum. Cajal × 47.
Fig. 2. Proliferation of astrocytes in the mammillary body. H. & E × 150.
Fig. 3. Neuronophagia in the hippocampal gyrus. H & E × 140.
Fig. 4a. Chromatolysis with peripheral vacuolation in a neurone of the red nucleus H & E × 120.
Fig. 4b. Necrosis of neurone in the red nucleus. H & E × 150.
Fig. 5a. Vacuolation of neurones in the red nucleus. (Note intravacuolar bodies). H & E × 110.
Fig. 5b. Eosinophilic degeneration in a neurone of the dentate nucleus. (Note loss of structure in the nucleus). H & E × 280.
Fig. 6a. Eosinophilic degeneration in a Purkinje cell. (Note pyknosis of the nucleus). H & E × 350.
Fig. 6b. Granulovacuolar degeneration of a neurone in the gyrus subcallosus. H & E × 350.

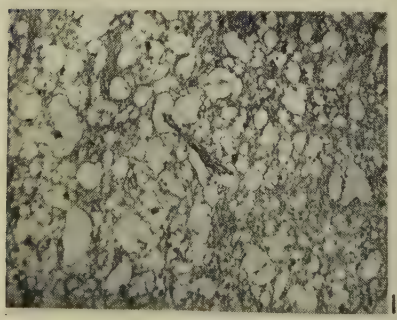
To face page 448











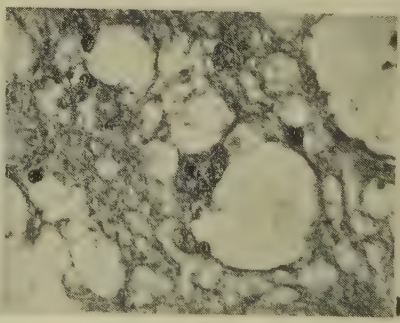
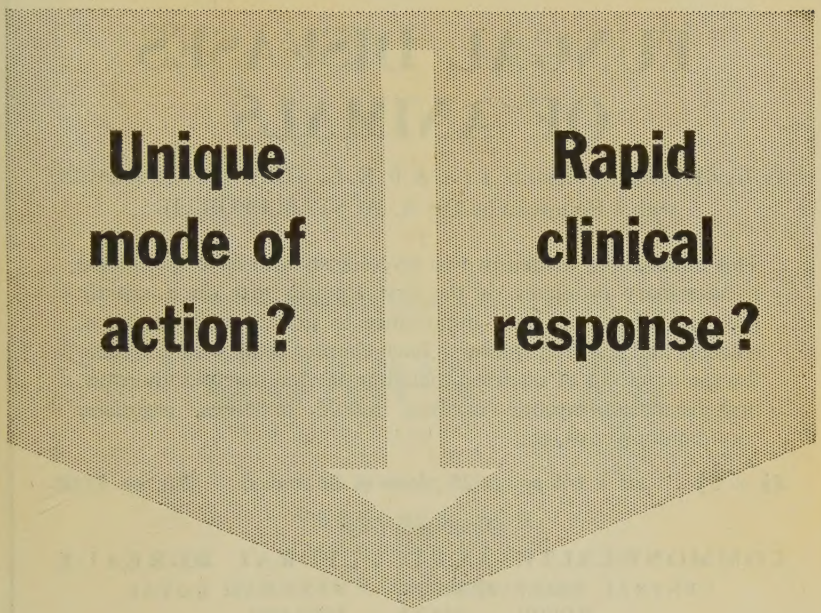


Fig. 7. Severe degeneration and vacuolation in the nucleus of the inferior olive of the medulla. H & E × 27.
Fig. 8. Severe vacuolation in neurones of the red nucleus. H & E × 38.
Fig. 9. Severe degeneration and vacuolation in the gyrus subcallosus. H & E × 110.
Fig. 10. Moderate vacuolation of the intercellular ground substance in the medial geniculate body. H & E × 180.
Fig. 11. Severe vacuolation of the intercellular ground substance. Thalamus. H & E × 27.
Fig. 12. Severe vacuolation of the intercellular ground substance. Thalamus. H & E × 180.



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